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**Platelet releasate promotes skeletal myogenesis by increasing muscle stem cell commitment to differentiation and accelerates muscle regeneration following acute injury**

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**Short title:** Platelet releasate & skeletal myogenesis

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**Abstract**

**Aim:** The use of platelets as biomaterials has gained intense research interest. However, the mechanisms regarding platelet-mediated skeletal myogenesis remain to be established.

The aim of this study was to determine the role of platelet releasate in skeletal myogenesis and muscle stem cell fate *in vitro* and *ex vivo* respectively.

**Methods:** We analysed the effect of platelet releasate on proliferation and differentiation of C2C12 myoblasts by means of cell proliferation assays, immunohistochemistry, gene expression and cell bioenergetics. We expanded *in vitro* findings on single muscle fibres by determining the effect of platelet releasate on murine skeletal muscle stem cells using protein expression profiles for key myogenic regulatory factors.

**Results:** TRAP6 and collagen used for releasate preparation had a more pronounced effect on myoblast proliferation versus thrombin and sonicated platelets ( $P < 0.05$ ). In addition, platelet concentration positively correlated with myoblast proliferation. Platelet releasate increased myoblast and muscle stem cell proliferation in a dose-dependent manner, which was mitigated by VEGFR and PDGFR inhibition. Inhibition of VEGFR and PDGFR ablated MyoD expression on proliferating muscle stem cells, compromising their commitment to

differentiation in muscle fibres ( $P < 0.001$ ). Platelet releasate was detrimental for myoblast fusion and affected differentiation of myoblasts in a temporal manner. Most importantly we show that platelet releasate promotes skeletal myogenesis through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis and accelerates skeletal muscle regeneration after acute injury.

**Conclusion:** This study provides novel mechanistic insights on the role of platelet releasate in skeletal myogenesis and set the physiological basis for exploiting platelets as biomaterials in regenerative medicine.

**Key words:** growth factors, platelet releasate, platelet-rich plasma, regeneration, skeletal muscle, satellite cells

## Introduction

Skeletal muscle is a remarkably plastic tissue with a strong capacity to regenerate after injury due to a small population of skeletal muscle stem cells, termed satellite cells. These muscle stem cells are typically quiescent, but become activated upon myofibre injury, proliferate and either return to quiescence or differentiate to support regeneration<sup>1</sup>. However, this regenerative ability typically comes at a cost, such as incomplete functional recovery, stiffness, excessive inflammation or fibrosis<sup>2-4</sup>. There is currently an increasing interest in the field of skeletal muscle regenerative research. This is driven by the increasing prevalence of injuries in both office and sporting vocations, a lack of effective treatments for myopathies, neuromuscular disease and sarcopenia, and finally, an intense interest in preventing skeletal muscle atrophy in microgravity<sup>5-8</sup>. Taking all these factors into consideration, skeletal muscle injuries, myopathies and research into these areas are of great economical interest to both governmental bodies and private-sector industries alike. At

present, conventional treatments to musculoskeletal injuries include rest, ice, compression and elevation (RICE), nonsteroidal anti-inflammatory drugs (NSAIDs) and physical therapy <sup>9</sup>.

Lacking evidence for the effectiveness of RICE and NSAIDs leave the field of skeletal muscle regeneration somewhat redundant <sup>10, 11</sup>. Autologous platelet rich plasma (PRP; an autologous biomaterial where platelets are re-suspended in plasma) treatment has emerged as an alternative to the above-mentioned methods. This is due to the cost, ease of availability to numerous cytokines and growth factors (such as IGF-1, VEGF and PDGF contained in the alpha-granules of the platelets) to the targeted area, acting as biomaterials to regulate cell cycle, promote wound healing and stimulate regeneration <sup>12-15</sup>. Growth factors and cytokines are essential components of the early inflammatory response and subsequent successful tissue regeneration <sup>16</sup>. However, there are inconsistencies in the literature as to whether PRP proves beneficial in skeletal muscle regeneration <sup>13, 17</sup>. It has been speculated that methodological variability may account for the observed discrepancies among studies on the effectiveness of PRP in wound healing and tissue regeneration <sup>13, 18, 19</sup>. Another leading proposition for the debatable effects of PRP on skeletal muscle regeneration may be the abundance of cytokines that can induce fibrosis, such as transforming growth factor beta-1 (TGF- $\beta$ 1) <sup>3, 4, 20-22</sup>.

Data from clinical studies on the effectiveness of PRP-based applications for skeletal muscle regeneration is limited. Therefore, the need for a better understanding of the effects of platelets on skeletal muscle regeneration is necessary. Recent data suggest that platelet releasate, a refined suspension of growth factors released from aggregated platelets, where the platelet cellular-debris is removed, can be used as an alternative method to using PRP for treating musculoskeletal regeneration <sup>15, 17, 23-25</sup>.

Therefore, the aim of this study was: i) to determine the impact of methodological variability in platelet preparations on skeletal myogenesis, ii) dissect the effect of platelet releasate on myoblast and muscle stem cell's myogenic potential *in vitro* and *ex vivo* respectively and iii) to define the molecular mechanisms of platelet releasate in regulating muscle stem cell fate and recruitment. Briefly, we show here that platelet releasate is capable to drive myoblast and muscle stem cell proliferation and differentiation both *in vitro* and *ex vivo*. This effect is mediated by the PDGF/VEGF pathway that promotes muscle stem cell commitment to differentiation and ultimately enhances myogenesis. The findings of this study set the basis for exploiting platelets as biomaterials to promote tissue regeneration in future studies.

## Results

**The effect of platelet agonists and centrifugation speed during releasate preparation and dosage of releasate on C2C12 myoblast proliferation.** There is currently large controversial evidence on the role of PRP in tissue regeneration<sup>13, 17-19, 26, 27</sup>. We therefore, aimed to determine the effect of methodological variations during platelet releasate preparation on C2C12 myoblast proliferation. To this aim, C2C12 myoblasts were cultured for 24 hours in serum-free media (SF) supplemented with releasate (R) derived from either collagen, TRAP6 (a PAR1 agonist), or thrombin-activated platelets, which were centrifuged at different speeds after stimulation (i.e. 1400, 5500 and 9500g) (**Figure 1A-C and S. Figure 1**). These were compared to cells incubated with lysates from sonicated platelets (i.e. S.L.). Treatment of cells with 10% releasate resulted in significantly increased cell proliferation (i.e. 32-55%) independent of the agonist and centrifugation speed as compared to SF-treated cells (i.e. 5%; negative control). C2C12 myoblast proliferation in response to treatment with TRAP6- and collagen-activated releasate was comparable to cells treated with 20% growth medium (GM, containing 20% FBS; positive control). However, the highest percentage for myoblast proliferation amongst groups was found for PAR1-activated platelet releasate independent of centrifugation speed. Obtaining platelet lysates by sonication did

not show any additional advantage on myoblast proliferation as compared to releasate activated with either TRAP6 or collagen. Of note, collagen-, TRAP6- and thrombin-activated platelets had similar levels of aggregation and total protein ( $\text{mg mL}^{-1}$ ) (**S. Figure 2, Figure 1D** respectively). Taken together, these findings suggest that growth factors from thrombin-activated or sonicated platelets improve C2C12 myoblast proliferation to a lesser extent than collagen- or PAR1-activated platelet releasate, while the final centrifugation speed during platelet preparation does not appear to play an important role. We therefore, continued using TRAP6 at 9500g to obtain platelet releasate for all subsequent experiments. The proliferative effect using this method of platelet preparation was supported with Ki-67 and cell viability experimentation (**S. Figure 3**).

We next determined the dose-response of platelet releasate on C2C12 cell proliferation. Cells were treated with 10-, 20- or 30% releasate for 24 hours. Administration of 10-30% releasate resulted in significant myoblast proliferation compared to serum free (SF) conditions (50-62% vs. 5% respectively; **Figure 1E**). In addition, 20- and 30% releasate exhibited a higher proliferative effect on murine myoblasts as compared to the 10% releasate group (**Figure 1E**). These data are indicative of a dose-dependent effect of releasate on myoblast proliferation.

**Human releasate has a stronger proliferative effect on C2C12 cells than murine releasate.** We also established the effect of human and mouse platelet releasate on myoblast proliferation. From an ethical point of view, human platelet releasate is more readily available without the need for murine sacrifice<sup>28</sup>. However, the species-specific variability of murine versus human platelet releasate treatment on murine C2C12 myoblast proliferation was investigated. We sought to establish whether using the same concentration of platelets ( $2.5 \times 10^8 \text{ mL}^{-1}$ ; physiological levels of platelets in human blood) of 10% murine platelet releasate (Mouse R) on murine cells would yield better proliferation than 10% human platelet releasate (Human R) due to species homology. Serum-free conditions and 20% FBS



(20% GM) were used as negative and positive controls respectively. Mouse releasate was found to be significantly less effective by 10% in promoting cell division than human releasate (**Figure 1F**). This may be accountable to the fact that nominal levels of platelets in murine blood are 3.5-7 times higher than human blood (i.e.  $12.5 \times 10^8 \text{ mL}^{-1}$ ; physiological levels of platelets in mouse versus  $2.5 \times 10^8 \text{ mL}^{-1}$  in human blood). In addition, the platelets of mice are considerably smaller in size (2-4 times) than human platelets<sup>29</sup>. Since murine platelets do not express the PAR-1 receptor, we used collagen as a platelet agonist<sup>30</sup>. Correcting for these variables may optimise the proliferative capacity of murine releasate.

**Releasate from physiological platelet concentrations is beneficial for myoblast proliferation.** We next aimed to explore the effect releasate from varying platelet concentrations on myoblast proliferation. To address this, platelet concentrations of  $0.1 \times 10^8$  –  $10 \times 10^8$  platelets  $\text{mL}^{-1}$  from human blood were used (**Figure 2A**). Releasate prepared from sub-physiological levels of platelets (i.e.  $0.1$ – $1 \times 10^8$  platelets  $\text{mL}^{-1}$ ) resulted in diminished myoblast proliferation compared to cultures supplemented with growth medium (GM; 10% FBS, positive control). However, releasate from physiological platelet concentrations (i.e.  $2.5 \times 10^8$  platelets  $\text{mL}^{-1}$ ) resulted in significantly higher myoblast proliferation compared to releasate from sub-physiological platelet concentrations and serum free conditions, which was similar to the growth medium (GM) group. There was a positive correlation between the platelet concentration used for releasate preparation and myoblast proliferation (**S. Figure 4**). Interestingly, releasate from supra-physiological levels of platelets exhibited additional effects on myoblast proliferation by approximately 30% compared to the physiological concentration. These findings indicate that the supra-physiological levels of platelets have a stronger effect on the proliferative capacity of the C2C12 myoblasts.

### **The effect of releasate on gene and protein expression patterns of C2C12 myoblasts.**

We next applied releasate from physiological levels of human platelets ( $2.5 \times 10^8$  platelets mL<sup>-1</sup>) on proliferating C2C12 myoblasts for 48 hours and we measured gene expression by quantitative real-time RT-PCR. There were significantly higher mRNA levels ( $p < 0.001$ ) for *Vegfa165*, *Vegfr1*, *Pdgfa*, *Pdgfb* (growth factors and their receptors involved in myogenesis), *Pax7*, *Myf5* and *Myod* (myogenic regulatory factors) and *Prmt1* (a regulator of muscle stem cell fate) in response to releasate treatment in serum-free or serum-rich conditions compared to non-releasate controls (i.e. SF and GM respectively). We observed significantly lower ( $p < 0.01$ ) mRNA levels for *Igf1* (growth factor involved in myogenic differentiation) in all groups as compared to the SF group. Transcript levels of the *Igf1r* and *Pdgfar* receptors were unchanged between groups. *Pdgfr* mRNA levels were upregulated by releasate in the GM group only (**Figure 2B**). mRNA levels for the DNA repair genes *Ogg1* (8-Oxoguanine glycosylase), *Gadd45g* (Growth arrest and DNA-damage-inducible protein 45 gamma) and *Parp1* (Poly [ADP-ribose] polymerase 1) were increased for all groups versus the SF group (**S. Figure 5**). Gene expression of neurotrophins and their receptors (i.e. Nerve growth factor; *Ngf*, Brain-derived neurotrophic factor; *Bdnf*, Neurotrophin-3; *Ntf-3* and their receptors Tropomyosin receptor kinase A, B and C; *TrkA*, *TrkB* and *TrkC* respectively) was not affected by the administration of releasate on proliferating myoblasts (**S. Figure 5**). Platelet releasate also induced higher PDGFB and VEGF protein levels in proliferating C2C12 myoblasts (**Figure 2C** and **S. Figure 6**). These findings suggest that platelet releasate regulates PDGF and VEGF mRNA and protein expression.

**Growth factors in platelet releasate.** We next aimed to get insights into the levels of relevant growth factors contained in platelet releasate. For this purpose, we stimulated washed platelets from human blood with agonists that induce platelet aggregation and degranulation [i.e. TRAP6 (a PAR1 agonist) or collagen] or left the platelet suspension untreated. The levels of 32 biomarkers involved in cell proliferation and differentiation were measured. All biomarkers were increased several-fold in TRAP6 and collagen induced

releasate compared to unstimulated platelets (**Figure 3**). In particular, we found that between unstimulated and stimulated (Collagen and TRAP6) groups, two clusters based on relative heatmap intensity have emerged. One cluster with a moderate increase including uPA, IL-18, Angiopoietin-2, Follistatin, IGFBP-1, Endoglin, IL-6, sCD40L, PLGF, TGF- $\alpha$  and TNF- $\alpha$  and a second cluster with >10-fold increase including VEGF-A, -C and -D, SCF, PDGF, EGF, sFASL, HGF, sVEGFR-1, -2, sTIE2, FGF and sEGFR. Lower levels of some biomarkers were detectable in unstimulated platelets. Published evidence regarding the role of these biomarkers in myoblast proliferation and differentiation is summarised in supplementary **Table 1**.

**Platelet releasate drives myoblast proliferation through PDGF and VEGF.** Given the above observations that mRNA and protein levels of PDGF and VEGF are increased in proliferating myoblasts (**Figure 2**), as well as their abundance in the releasate (**Figure 3**), we reasoned that these growth factors may be governing cell proliferation, as shown when individually targeted<sup>31, 32</sup>. To further investigate this notion, we used low, medium and high doses of VEGFR or PDGFR Inhibitors with or without releasate to assess C2C12 cell proliferation (**Figure 4A, B**). Treatment with VEGFR and PDGFR inhibitors resulted in a dose-dependent attenuation of myoblast proliferation in groups treated with platelet releasate (**Figure 4B**). A similar trend was observed under serum free (SF) conditions with the VEGFR and PDGFR inhibitors. Additionally, VEGFR and PDGFR inhibition compromised C2C12 proliferation under serum-rich (i.e. growth medium) conditions as indicated by decreased total cellular number (**S. Figure 7**). Taken together, these data suggest that myoblast proliferation is, at least in part, driven by the VEGF and PDGF growth factors contained in the platelet releasate.

**Temporal effects of platelet releasate on myoblast differentiation.** It is known that growth factors contained in platelet granules such as Hepatocyte growth factor (HGF), TGF- $\beta$ 1, Insulin-like growth factor 1 (IGF-1), Soluble Angiopoietin-1 receptor (sTIE-2), Angiopoietin-2 and Vascular Endothelial Growth Factor Receptor-1 (sVEGFR-1) are beneficial for myotube fusion<sup>3, 15, 22, 33, 34</sup>. Therefore, we next hypothesised that releasate would be beneficial in C2C12 myoblast differentiation into myotubes. To test this hypothesis, we assessed myotube differentiation in response to releasate administration either i) during the proliferative phase (day 0-2, GM+R;DM) or ii) throughout the proliferation and differentiation (day 0-9, GM+R;DM+R). We found a significant decrease in myogenic fusion and differentiation mRNA (*Myogenin*, *Mhc1*, *Mhc2a*, *Mhc2b*, *Acta1*, *Tmem8c*, *Bex1* and *Igf1*) for the continuously added releasate group (GM+R;DM+R) versus the other two groups (GM;DM and GM+R;DM; which were non-significantly different for all expression). *Myod*, *Pgdf $\beta$*  and *Vegfa165* mRNA expression was increased in all groups that had received releasate treatment. *Pax7* mRNA expression was decreased for GM+R;DM and increased for GM+R;DM+R versus the GM;DM positive control group (**Figure 5A**). Strikingly, continuous administration of releasate during day 0-9 resulted in a robust inhibition of myoblast differentiation as shown by impaired myofusion, myotube number, myogenin expression, myotube length and myotube thickness with increased nuclei number. Subsequently, we asked whether platelet releasate improves myotube terminal differentiation when administered after the initiation of myoblast fusion (days 5-12, GM;DM+R.D5-12). We found that addition of releasate during terminal differentiation resulted in significantly increased total nuclear number, myotube number and myogenin expression without affecting the myofusion index or the myotube length and thickness (**Figure 5B**). Taken together, these findings suggest that continuous administration of releasate causes myoblasts to fail exiting the proliferative phase and has detrimental effects on myotube formation. Conversely, administration of platelet releasate after the initiation of myotube formation (i.e. terminal differentiation) is beneficial for myoblast differentiation.

**PDGFR and VEGFR inhibition reduces myoblast differentiation.** To gain mechanistic insights into the role of releasate on myoblast differentiation, we treated differentiating myoblasts with PDGFR and VEGFR inhibitors with or without platelet releasate (**Figure 6A**). We found that PDGFR inhibition reduced the total nuclei number, myogenin expression and total myotube number in cultures with or without platelet releasate. Similarly, VEGFR inhibition during myoblast differentiation resulted in decreased total nuclei number, myogenin expression and myotube number in cultures with or without platelet releasate (**Figure 6B-C**). These results indicate that PDGF and VEGF contained in releasate both are important for terminal myoblast differentiation.

**Platelet releasate increases myofibre stem cell commitment to differentiation through VEGF and PDGF in a dose-dependent manner.** We next sought to determine if platelet releasate affected muscle stem cell expression profiles by using the single fibre *ex vivo* model. Immunohistochemical detection of Pax7<sup>+</sup>/MyoD<sup>-</sup>, Pax7<sup>+</sup>/MyoD<sup>+</sup> and Pax7<sup>-</sup>/MyoD<sup>+</sup> cells identified quiescent, activated/proliferating and committed-to-differentiation muscle stem cells, respectively (**Figure 7A**). Platelet releasate added 1-3 times (x) with media change within 48 hours of culture resulted in a dose-dependent increase of Pax7<sup>-</sup>/MyoD<sup>+</sup> cells compared to control (i.e. either serum free or fibre media conditions; **Figure 7B**). For example, treatment of fibres with platelet releasate x3 resulted in a 300-400% increase of Pax7<sup>-</sup>/MyoD<sup>+</sup> cells in either serum free or fibre media (i.e. serum rich) conditions, indicating that platelet releasate increased muscle stem cell commitment to differentiation. Furthermore, administration of releasate three times (x3) during a 48-hour culture induced a 50-100% increase in committed stem cells (i.e. Pax7<sup>-</sup>/MyoD<sup>+</sup>) as compared to a single addition of platelet releasate (x1). The commitment index, defined as the number of Pax7<sup>-</sup>/MyoD<sup>+</sup> cells per single fibre, was increased with releasate addition compared to serum free conditions and releasate supplemented with fibre medium had an additional cumulative effect (**Figure 7C**). Interestingly, inhibition of the VEGF and PDGF receptors independently reversed the effect of releasate on both the number of committed

stem cells (i.e. Pax7<sup>ve</sup>/MyoD<sup>ve</sup>) to control levels (i.e. fibre media) as well as the total stem cell number to serum free conditions (**Figure 7D**). In an attempt to identify key downstream factors involved in muscle stem cell progression, we targeted Cyclin D1 and Scrib, which have been acknowledged as two important regulators of cell cycle and muscle stem cell fate respectively<sup>24, 35</sup>. We found that platelet releasate resulted in a significant co-localisation of Cyclin D1 and Scrib with MyoD on muscle stem cells respectively, as well as a robust expression in proliferating C2C12 cells (**Figure 8A-C and S. Figure 8**). Ultimately, administration of platelet releasate on single fibres resulted in significantly higher muscle stem cell progeny independent of the use of serum in culture (i.e. SF and FM groups; **Figure 8D**). Collectively, these data suggest that use of platelet releasate has a powerful effect on driving muscle stem cell commitment to differentiation and similar to C2C12 cells, the effects of releasate are mediated by the VEGF and PDGF pathways through cell cycle and cell fate regulation.

**Platelet releasate increases the total number of Myogenin<sup>ve</sup> muscle stem cells and clusters per fibre.** We followed up single myofibre cultures at 72 hours, where muscle stem cell progeny is expected to have differentiated to give rise to new myonuclei driven by high expression of myogenin<sup>1</sup>. We found significantly increased Pax7<sup>ve</sup>/Myogenin<sup>ve</sup> cells in the releasate group as compared to serum free conditions, without any change in the relative expression patterns among groups (**Figure 9A-B**). The relative expression of Pax7<sup>ve</sup>/Myogenin<sup>ve</sup> cells (i.e. quiescent muscle stem cell pool) was not affected by the use of releasate, however was significantly increased between the FM and SF group. In addition, the total progeny stem cell number and the number of cell clusters per fibre were significantly higher in the fibre media and platelet releasate groups as compared to serum free media (**Figure 9B**). These data provide convincing evidence that the use of platelet releasate is beneficial for muscle stem cell differentiation *ex vivo*. Crucially, the normalised standardised rank curves illustrating the distribution of total muscle stem cell progeny number per myofibre were similar between the fibre medium (FM) and releasate (R) groups

as compared to serum free (SF) conditions after 48- and particularly 72- hours in culture. Furthermore, regression analysis correlating 48-hour and 72-hour muscle stem cell progeny numbers revealed different gradient values (i.e. slopes) indicating the muscle stem cell proliferation rates were different between SF cultures versus FM and releasate conditions (**Figure 9C**). These results suggest that the muscle stem cells that developed under the influence of releasate treatment retained their normal capacity to proliferate. Taken together, these findings suggest that platelet releasate promotes myogenesis to a similar extent as found for standard serum-rich cultures.

**Platelet releasate increases mitochondrial respiration of C2C12 myoblasts and skeletal muscle stem cells.** Since mitochondria play a central role in the regulation of cell proliferation, we aimed to determine whether the use of platelet releasate affects the bioenergetic capacity of C2C12 myoblasts and muscle stem cells, by measuring the OCR<sup>36, 37</sup>. We found significantly increased OCR in C2C12 myoblasts under serum free conditions but no change in serum rich cultures (**Figure 10A**). Conversely, we found significantly higher spare respiratory capacity in muscle stem cells supplemented with platelet releasate in either serum-free or -rich conditions (**Figure 10B**). These data indicate that platelet releasate robustly affects the bioenergetics of muscle stem cells.

**Platelet releasate accelerates regeneration of skeletal muscle following acute damage with cardiotoxin.** Having shown that platelet releasate had a profound effect on myoblast proliferation and differentiation, we next examined its impact on the regeneration of adult mouse skeletal muscle, a process that is dependent on the generation of muscle precursors and their differentiation from the resident muscle stem cells. To this end the impact of platelet releasate was determined on muscle that has been acutely damaged using cardiotoxin (CTX) which causes muscle fibre necrosis, followed by macrophage-mediated debris clearance. Subsequent formation of new muscle fibres originates from the progeny of satellite cells, which undergo proliferation, differentiation and fusion. Newly formed

regenerating muscle fibres were identified by their expression of embryonic myosin heavy chain (eMHC). We found that generation of newly formed fibres was significantly enhanced ( $P<0.001$ ) in mice that had been treated with releasate compared to those that received PBS (**Figure 11A**). Furthermore, we found a profound impact of platelet releasate on the clearance of dead and dying fibres. The number and size of dying fibres (identified through the infiltration of IgG into fibres) present at 5 days after CTX damage in PBS-treated mice was significantly higher than those treated with platelet releasate ( $P<0.001$ , **Figure 11B**). These results show that platelet releasate promotes muscle regeneration by acting not only on muscle cells but possibly other components of the regeneration process.

## Discussion

Platelet-based applications have gained much attention recently as an effective way to promote muscle regeneration reviewed by us and others <sup>13, 18, 19</sup>. However, data are inconsistent and we have currently a limited understanding of the molecular events that govern skeletal myogenesis using platelet-based products as biomaterials <sup>4, 17, 23</sup>. Due to largely inconsistent protocols in platelet preparation, we hypothesised here that methodological variables such as platelet agonists, final centrifugation speed and platelet count may be crucial in determining experimental outcomes among studies <sup>3, 24, 38, 39</sup>. Therefore, we determined the effect of platelet agonists and final centrifugation speed and species-of-origin during releasate preparation on C2C12 myoblast proliferation. We found increased myoblast proliferation in response to releasate administration, which is in line with previously published data on the use of platelet releasate <sup>20, 23, 24, 38, 40</sup>.



However, our data also suggest that platelet releasate from collagen and TRAP6, two widely used platelet agonists, have a stronger effect on myoblast proliferation as compared to thrombin or mechanically lysed platelets by sonication, independent of the final centrifugation speed used. This finding may be explained by possible detrimental effects of proteases on releasate growth factors, since thrombin itself is a serine protease, as well as digestive enzymes released in the ablation of sonicated platelets<sup>41, 42</sup>. In addition, the use of 10-30% of releasate resulted in a dose-dependent increase of myoblast proliferation, possibly due to higher amounts of releasate components. Since human platelet releasate was more powerful at promoting myoblast proliferation than mouse releasate, we used human releasate for all of the subsequent experiments. Platelet concentration was previously recognised as a potentially important variable for the diverse and conflicting data of PRP or releasate on tissue regeneration<sup>13, 43</sup>. Our data indicate that there is a linear relationship between platelet concentrations used for the production of releasate and myoblast proliferation and have to be taken into consideration in future studies. Collectively, despite a positive role of platelet releasate on myoblast proliferation, our data suggest that methodological variations such as: i) platelet agonists, ii) amount of releasate administered, iii) species of platelet origin and iv) platelet concentration may account for some of the discrepancies among studies. In order to be consistent with the amount of releasate used in several previous studies, we used physiological levels of platelets for the production of releasate in all the subsequent experiments<sup>3, 12, 20, 23, 44</sup>.

We next sought to determine the effect of releasate on myoblast gene and protein expression for factors known to regulate myogenesis. Gene and protein expression data from proliferating C2C12 myoblasts revealed a consistent significant increase in PDGF and VEGF in response to human releasate as compared to serum free (control) conditions. The physiological effect of PDGF on cell growth and C2C12 myoblast proliferation has been recognised previously<sup>45, 46</sup>. In fact, PDGF is found as homodimers of AA, BB, CC and DD or

a heterodimer of AB isoforms. Although PDGF-AA and PDGF-AB have little or no effect on myogenic proliferation and differentiation, it has been shown that PDGF-BB promotes muscle stem cell proliferation but inhibits differentiation<sup>47-49</sup>. In turn, VEGF165 $\alpha$  has been shown to enhance C2C12 myoblasts migration and exhibit anti-apoptotic effects<sup>50</sup>. Higher mRNA levels of *Pax7*, *Myf5* and *Myod* in proliferating C2C12 myoblasts in response to releasate treatment may be part of a coordinated response of myogenic regulatory factors that are known to promote myogenic population expansion<sup>51</sup>. This is in line with previous evidence showing failure to withdraw from the cell cycle and commit to differentiation in response to releasate use<sup>24</sup>. Impaired *Igf1* mRNA levels followed by unchanged mRNA and protein levels of IGF1R $\alpha$  indicate that the effect of releasate on proliferating myoblasts is not mediated by IGF1. IGF1 is known to increase embryonic myoblast proliferation, but *Igf1* mRNA levels tend to be higher during myogenic differentiation<sup>46, 52</sup>. Increased mRNA levels of *Prmt1* in response to releasate administration are in line with previous data reporting that arginine methylation by *Prmt1* regulates muscle stem cell fate and it is important for myoblast fusion<sup>53</sup>. Taken together, these data suggest that the observed effects on myoblast proliferation by platelet releasate may be at least in part mediated by the PDGF and VEGF growth factors.

Given that *Ngf* has been shown previously to affect myogenic cell proliferation and differentiation and its expression is induced by platelet-rich plasma, we sought to determine whether platelet releasate affects neurotrophic growth factors (e.g. *Ngf*, *Bdnf*, *Ntf-3*, *TrkA*, *TrkB* and *TrkC*) gene expression in proliferating myoblasts<sup>54, 55</sup>. Our data suggest that administration of releasate on proliferating myoblasts does not upregulate the gene expression of neurotrophic growth factors. However, one has to bear in mind that neurotrophic factors and their receptors are more important for myogenic differentiation and their expression patterns in myotubes in response to platelet releasate remains to be established.

By using a multiplex immunoassay we quantified several growth factors in human platelet releasate in response to known platelet agonists (i.e. TRAP6 and collagen). The heatmap of those factors suggests that their amount increases several-fold in platelet releasate resulting in two clusters of moderate and higher intensity respectively. Of note, the factors SCF, FGF- $\beta$ , HGF, HER2, Follistatin, VEGFR-1, EGFR, PDGF-AB and PDGF-BB (known to regulate proliferation<sup>47, 48, 56-63</sup>) and VEGFR-2, IL-6, TIE-2 and HB-EGF (known to induce differentiation<sup>61, 64-67</sup>) showed a 4-20-fold increase in the TRAP6-activated platelet releasate as compared to unstimulated platelets. EGF, FGF, VEGF and PDGF-BB have been shown to promote myoblast proliferation and consistently inhibit myogenic differentiation, while VEGFR2 expression is increased during differentiation<sup>48, 57, 61, 63, 68, 69</sup>. Moreover, SCF increases skeletal muscle stem cell number, IL-6 regulates myoblast proliferation and migration, while TNF- $\alpha$  inhibits myogenic differentiation in C2C12 cells through NF- $\kappa$ B and promotes myotube atrophy<sup>56, 64</sup>. Conversely, IGFBP-1, Angiopoietin-2 and uPA have been shown to promote myogenic differentiation<sup>57, 70, 71</sup>. Of note, we cannot rule out that other growth factors (not included in the Bioplex kits used in this study) contained in the platelet releasate may potentially affect myogenesis. Taken together these data suggest the numerous growth factors, cell cycle regulators and transcription factors present in platelet releasate can be used to promote skeletal myogenesis.

We next aimed to determine the role of PDGF and VEGF contained in the human platelet releasate in skeletal myogenesis. For this reason, we cultured C2C12 myoblasts with releasate and administered either a PDGFR or VEGFR known inhibitor<sup>72, 73</sup>. Our data show a dose-dependent attenuation of myoblast proliferation for both inhibitors used separately, independent of the use of platelet releasate. These findings suggest that the powerful proliferative effect of platelet releasate on C2C12 myoblasts is, at least in part, mediated through PDGF and VEGF signalling. The proliferative effect of releasate is abolished by the highest dosage of PDGF or VEGF inhibitors. This may possibly be due to a crosstalk with

other growth factor receptors present in the releasate such as EGFR when used in high concentrations<sup>74</sup>. This notion is strengthened by previous studies showing that PDGF/VEGF inhibition resulted in diminished myoblast proliferation and recombinant PDGFs improved skeletal muscle recovery after injury<sup>20, 31, 47-50, 61, 75, 76</sup>.

In contrast to myoblast proliferation, the use of platelet releasate during myogenic differentiation seems to be more complex. We show here that, on one hand, treatment of myotubes with releasate during terminal differentiation promotes myogenic differentiation (i.e. myogenin<sup>+</sup> cells and myotube number). On the other hand, administration of platelet releasate during both proliferation and differentiation of C2C12 myoblasts results in attenuated differentiation based on largely impaired *Myogenin*, *Mhc1*, *Mhc2a*, *Mhc2b*, *Acta1*, *Tmem8c*, *Bex1*, *Igf-1* mRNA, myogenin protein expression, myotube number, myofusion index, myotube length and thickness, which is in line with recent studies using platelet-rich plasma<sup>3, 22, 40</sup>. This is indicative of a failure to withdraw from the cell cycle, supported by the increased nuclei number (**Figure 5A**). Importantly, we provide evidence that platelet releasate is beneficial for myogenic differentiation leading to higher myogenin expression and larger myotubes, when it is administered after the early differentiation phase, where myoblast fusion into myotubes has started. Taken together, these data suggest that the timing of application of releasate to C2C12 myoblasts or myotubes is crucial and may account for discrepancies in the literature over whether releasate hampers or improves differentiation<sup>17, 21, 44</sup>.

Similar to myoblast proliferation, inhibition of PDGFR and VEGFR resulted in attenuated C2C12 myotube differentiation as shown by impaired myogenin expression and myotube number independent of releasate administration. This finding highlights the importance of PDGF and VEGF during myogenic differentiation. Previous research data suggest that

VEGF is important for myogenin expression as well as myoblast and myotube number<sup>31, 69</sup>. Moreover, addition of PDGF-BB to myoblasts inhibits differentiation<sup>32, 47, 48, 76</sup>. Of note, the myotube fusion index did not significantly differ amongst groups, indicating proliferation factors may be necessary for proper differentiation capacity and that lower total nuclei number may be accountable for the attenuated differentiation. Therefore, it appears that PDGF plays an important role during myoblast differentiation, which is in line with previous evidence that PDGFR inhibition decreases muscle mass<sup>75</sup>.

Collectively, we have shown so far that platelet releasate is capable of promoting myoblast proliferation and exhibits opposing effects on myogenic differentiation dependent on the time of application. We have also highlighted the important role of PDGF and VEGF for both myogenic proliferation and differentiation. This is important since PDGF and VEGF pathways interact through the Akt/mTOR pathway to induce skeletal muscle stem cell proliferation<sup>49, 77, 78</sup>. In order to determine the effect of platelet releasate on muscle stem cell proliferation and differentiation profiles we used murine single muscle fibres in culture. We show here for first time that platelet releasate administration on single myofibres results in significant increase of Pax7/MyoD<sup>+</sup> muscle stem cells in a dose-dependent manner. These findings depict an increased commitment of proliferating muscle stem cells to differentiation as shown by MyoD<sup>+ve</sup> cells per total myonuclear number (i.e MyoD+/DAPI/fibre). The increase in committed muscle stem cells to differentiation in response to platelet releasate is evident for both serum free and standard fibre growth conditions, indicating that releasate may act as an appropriate substitute for the regular fibre growth medium. To this end, several studies have aimed to replace bovine serum by autologous platelet-based applications<sup>20, 40</sup>.

Although upregulation of MyoD mRNA and protein in rat skeletal muscle stem cells by PRP has been shown previously on muscle sections, we provide here evidence that this is taking place with the concomitant downregulation of Pax7 which results in higher number of stem cells following the differentiation pathway to support myofibre regeneration<sup>44, 45</sup>. Importantly, the effect of platelet releasate on MyoD is reversed by PDGFR and VEGFR inhibition, indicating a possible crosstalk between these factors. Indeed, addition of PDGF-BB decreases myoblast fusion, however it upregulates MyoD during myogenesis<sup>32</sup>. Interestingly, application of platelet releasate resulted in a robust increase of Cyclin D1 and Scrib expression on muscle stem cells. Cyclin D1 driven by platelet releasate is known to regulate the cell cycle and is important for cell proliferation<sup>24</sup>. Most importantly, Scrib is a significant regulator of myogenic progression after commitment to differentiation that dictates muscle stem cell fate and is indispensable for muscle repair<sup>35</sup>.

It has been claimed that the beneficial effects of platelet rich plasma on myogenesis can be brought about by the plasma per se<sup>3</sup>. However, this possibility can be ruled out, since our data indicate that the use of platelet poor plasma does not mimic the findings on muscle stem cells observed with platelet releasate. Beyond that, platelet preparations were conducted with washed platelets in modified tyroid's buffer and not plasma in this study.

Despite previous evidence on compromised myogenic differentiation in response to PDGF administration, we found that platelet releasate was beneficial for muscle stem cell differentiation<sup>32</sup>. In particular, we report that platelet releasate on single muscle fibres leads to significantly higher expression levels of Myogenin in differentiated muscle stem cells, significantly higher stem cell progeny and total stem cell numbers as well as number of stem cell clusters per fibre. Further to this, isolated primary skeletal muscle stem cells have an increased number, fusion index and myotube number when co-cultured with platelet

releasate and growth medium simultaneously; mitigating the negative effects that releasate has on differentiation alone. Altogether these findings provide novel evidence on a powerful effect of platelet releasate on muscle stem cell proliferation and commitment to differentiation through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis (**Figure 10D**).

In order to obtain insights in the metabolic homeostasis of proliferating cells exposed to platelet releasate, we measured the oxygen consumption rate at the cellular level. We found that administration of releasate resulted in significantly higher spare oxidative capacity in isolated muscle stem cells under either serum free or serum rich conditions. This finding indicates that platelet releasate may alter metabolic homeostasis of muscle stem cells, which is important for myogenesis and most importantly for muscle regeneration. This notion is supported by previous studies showing that cardiac myoblasts (i.e. H9C2 cells) had an increased respiratory reserve capacity in response to platelet-rich plasma <sup>37</sup>. In the current study, platelet releasate did not upregulate the OCR of C2C12 myoblasts under serum-rich conditions, as opposed to primary murine stem cells. A possible explanation of this finding may originate in the recently described role of MyoD in regulating skeletal muscle oxidative metabolism with implications for energy availability and muscle contraction<sup>79</sup>. Evidence also suggests that reactive oxygen species (ROS) are essential for activating several growth factors including PDGFs <sup>80</sup>. Increased ROS production may overwhelm the antioxidant capacity of the cell resulting in oxidative stress. It can be speculated that the increased mRNA levels of DNA repair enzymes (i.e. *Ogg1*, *Gadd45g* and *Parp1*) in response to releasate treatment reported in this study may be part of a cellular response to the enhanced energetic state and potentially increased stress during accelerated myoblast proliferation.

Skeletal muscle regeneration following acute damage is a process that is reliant on a number of cell types that coordinate their activity to reconstitute a working tissue<sup>81</sup>. This includes not only the resident stem cell population (i.e. satellite cells), which regenerate most if not all the muscle cells that eventually fuse to form muscle fibres but also macrophages and angioblasts<sup>82-84</sup>. Here we show that platelet releasate promotes muscle regeneration following cardiotoxin injection evidenced the presence of larger newly formed muscle fibres. This outcome is in keeping with our in-vitro data in this paper showing that platelet releasate impacts on the proliferation and differentiation of myoblasts. Herein the accelerated regeneration is postulated to be a consequence of direct impact of the release on satellite cells. An additional possibility is that the angiogenic factors in the releasate could act on satellite cells indirectly so that that firstly promote angiogenesis in the regenerating tissue which then impacts on the activity of satellite cells. Indeed, angiogenesis is a major factor in muscle regeneration and can even compensate to a large decrease in muscle stem cells and still deliver robust regeneration<sup>85</sup>. Finally, we show that the number of dying fibres and their size (an indicator of the clearance process) was greatly reduced by platelet releasate, a process controlled by macrophages. This may imply a role for the platelet releasate in controlling the pro-inflammatory/anti-inflammatory properties of macrophages. This could be of particular importance to setting of chronic muscle damage in a clinical context (such as Duchenne Muscular Dystrophy) in which the inflammatory process induces fibrosis and attenuates muscle regeneration<sup>86</sup>.

In conclusion, the present study provided evidence that methodological variability may account for discrepancies among studies on the role of platelet releasate in skeletal muscle regeneration. Platelet releasate promotes myoblast proliferation and terminal differentiation both *in vitro* and *ex vivo*. However, the timing of releasate application appears to be critical due to the inhibitory role of releasate on myoblast fusion as shown here and elsewhere<sup>40</sup>. Most importantly, we showed for the first time that platelet releasate increases muscle stem



cell commitment to differentiation and promotes skeletal myogenesis through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis and accelerates skeletal muscle regeneration after acute injury. Collectively, the present findings can be exploited in future studies to pinpoint the role of platelets as biomaterials in skeletal muscle regeneration.

## Methods

**Ethics statement.** The study was approved by the local Ethics Committee of the University. The animal experiments were performed under a project license from the United Kingdom Home Office in agreement with the revised Animals (Scientific Procedures) Act 1986 and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). Blood sampling from healthy human volunteers was performed with their written informed consent and was approved by the University's Ethics Committee.

**Preparation of platelet releasate.** Human platelet releasate (R) was prepared in acid citrate dextrose (ACD) to whole blood at a ratio of 1:5, centrifuged at 190g for 15 minutes followed by PRP collection and inactivation of platelets using prostaglandin I<sub>2</sub> (534nM; Cayman Chemical). The PRP was then centrifuged in a swing-out rotor at 800g for 12 minutes and the platelet-poor plasma (PPP) supernatant was then removed. Modified Tyrode's buffer (NaCl, HEPES, NaH<sub>2</sub>PO<sub>4</sub>, NaHCO<sub>3</sub>, KCl, MgCl<sub>2</sub> and D-Glucose) was used to re-suspend the platelet pellet to a concentration of  $2.5 \times 10^8$  platelets mL<sup>-1</sup> (unless otherwise stated) using a cell counter (Beckman Coulter; Z1-Series Coulter® Particle Counter). The platelet preparation was activated using a PAR1 agonist (TRAP6; 20μM; AnaSpec; cat. AS-60679), thrombin (0.05-0.1 NIH Units mL<sup>-1</sup>; Sigma Aldrich; cat.9002-04-4), or collagen (10μg mL<sup>-1</sup>; BioData; cat. 101562), in an aggregometer (CHRONO-LOG® Model 490 4+4 Optical Aggregation System, USA). Alternatively, after counting and re-suspending

in modified Tyrode's buffer, platelets were sonicated for 5 minutes using a 0.5 cycle at a gradually increasing amplitude of 60–100% (Hielscher Ultrasonic Processor - UP200S). Platelets were centrifuged at 9500g for 10 minutes, unless otherwise stated (see **S. Figure 1**), and the releasate supernatant was aliquoted and stored at -80 °C until further use.

Mouse platelets were prepared with 200µL ACD in a 1mL syringe & 25-gauge needle, gently mixed before transferring to 500µL modified Tyrode's buffer with 500µL ACD, per mouse, and centrifuge in a swing-out rotor at 100g for 5 minutes with no breaks. The PRP supernatant was then transferred using a Pasteur pipette to a clean Eppendorf tube and centrifuged at 800g for 5 minutes with no breaks. The platelet-poor plasma was discarded and platelets were resuspended in modified Tyrode's buffer to a concentration of  $2.5 \times 10^8$  platelets  $\text{mL}^{-1}$  using a Beckman Coulter particle counter. Collagen ( $10 \mu\text{g mL}^{-1}$ ) was used to activate the platelets in an aggregometer for 5 minutes at 37°C (reaching > 80% aggregation) before centrifuging at 9500g and storing releasates at -80 °C.

**Cell cultures and treatments.** Murine C2C12 skeletal myoblasts (American Type Culture Collection, VA) were cultured in growth medium (GM); Dulbecco's Modified Eagle's Medium (DMEM; HyClone (High glucose, no sodium pyruvate)) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich), 1% penicillin/streptomycin (PS; Sigma-Aldrich) and 0.1% amphotericin B (AB; Sigma-Aldrich) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. To induce differentiation, C2C12 cells were cultured in GM until reaching 80% confluence before switching to differentiation media (DM), containing DMEM plus 2% horse serum (HS; Gibco) 1% PS and 0.1% AB for either 6, 9 or 12 days. Releasate was added either once during proliferation at time-point 0, or at every media change (either every 12 hours or 24 hours depending on the experiment) during differentiation, unless otherwise stated. Inhibitors were added during DM media changes when indicated using 5µM PDGFR Inhibitor or a 130nM VEGFR Inhibitor. The myofusion index was calculated as myogenin<sup>+</sup> cells per

myotube (with a minimum threshold of 3 nuclei per myotube) divided by DAPI-stained cells as a percentage. For proliferation in all experimental groups, unless otherwise stated, C2C12 cells were cultured in serum free (SF) conditions (DMEM, 1% PS and 0.1% AB). Inhibitors used for proliferation and differentiation experiments were VEGFR Inhibitor (AAL-993; 23nM, 130nM or 1.30μM, Merck) and PDGFR Inhibitor (Tyrphostin AG 1295 [AG-1295]; 250nM, 500nM or 5μM, Santa Cruz).

**Cell proliferation and viability analysis.** C2C12 myoblast cell proliferation was also evaluated by the pyrimidine analogue EdU incorporation assay using the fluorescent Click-iT® EdU Cell Proliferation Assay (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Proliferating cells were measured as EdU divided by DAPI (4',6-diamidino-2-phenylindol-stained (Dako))-stained nuclei as a percentage. Cellular viability was assessed by seeding 50,000 C2C12 cells in a 35mm petri dish for 24 hours in either serum-free, 10% FBS growth medium, 10% releasate or 10% FBS growth medium plus 10% releasate, and counting the ratio of live to dead cells using trypan blue.

**Single fibre isolation and culture.** For single-fibre culture, murine C57Bl/6J single fibres were isolated from the extensor digitorum longus muscle (EDL). Limb muscles were dissected and subjected to collagenase (0.2%; Sigma Aldrich; cat. C2674) digestion for 3-4 hours at 37 °C and 5% CO<sub>2</sub> as described previously<sup>87</sup>. The digested muscle was then gently triturated with a Pasteur pipette. Fibres were then cultured for 48 and 72 hours in single fibre media (FM; 10% horse serum, 0.5% chick embryo extract), or serum free media (SF) with the addition of 10% releasate (R) 3 times during culture every 16 hours (unless otherwise stated). Fibres were stained for mouse monoclonal anti-Pax7 (1:200, Santa Cruz; cat. sc-81648), rabbit polyclonal anti-MyoD (1:200; Santa Cruz; cat. sc-760) and rabbit polyclonal anti-Myogenin (Santa Cruz; cat. sc-576), or with anti-Cyclin D1 (1:200 Santa

Cruz; cat. sc-450) or Scrib (Santa Cruz; cat. sc-374139) and with DAPI. The commitment index was calculated as MyoD-stained muscle stem cells divided by DAPI-stained cells as a percentage. PDGF and VEGF were inhibited in the myofibres using either a 5 $\mu$ M PDGFR Inhibitor or a 130nM VEGFR Inhibitor.

**Satellite cell isolation from single fibres.** For primary myoblasts, satellite cells (i.e. primary muscle stem cells) were derived from the EDL of wild-type C57Bl/6J mice. Briefly, limb muscles were dissected and subjected to 0.2% collagenase digestion for 4 hours at 37 °C. The digested muscle was then gently transferred to 35mm petri dishes where the collagenase was removed. Single muscle fibres were isolated in SF culture medium, by means of a gentle mechanical trituration with a Pasteur pipette and then cultured for 72 h in Matrigel (1mg mL<sup>-1</sup>; Corning Matrigel; cat. 354234) –treated 6-well plates, at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>, in satellite proliferation media containing DMEM, 30% FBS, 1.5% chick embryo extract (C.E.E.) plus 1% penicillin-streptomycin. Next, the myofibres were removed and the satellite cells were cultured as per experimental conditions. Differentiation of primary satellite cells (stem cells) was achieved through culturing for 3 days in appropriate proliferation medium as per experimental condition before switching to differentiation medium (5% Horse Serum, 0.5% C.E.E., 1% P.S. and 0.1% A.B.) for a further 5 days.

**Immunohistochemistry.** C2C12 cells or primary muscle stem cells as appropriate were seeded on coverslips in 1mL of media in 24-well plates (Corning® Costar® TC-Treated 24-Well Plates). Media was removed at the end of experiments with 4% paraformaldehyde added for 15 minutes, followed by two washes in phosphate-buffered saline. Permeabilisation buffer was then added for 20 minutes followed by two washes in wash buffer before applying onto optical slides. Primary antibodies for anti-Pax7 (Santa Cruz, cat. Sc-81648), anti-MyoD (Santa Cruz; cat. Sc-760), anti-Myogenin (Santa Cruz; cat. sc-52903),

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mouse monoclonal anti-IGF-1R $\alpha$  (Santa Cruz; cat. sc-271606), anti-PDGF B (Santa Cruz; cat. sc-365805), anti-VEGF (Santa Cruz; cat. sc-7269), anti-Ki-67 (ThermoFisher. Cat. 14-5698-80), anti-myosin heavy chain 3 (Santa Cruz; cat. sc-53091), anti-Cyclin D1 (Santa Cruz; cat. sc-450) or anti-Scrib (Santa Cruz; cat. sc-374139) were added (1:200 in wash buffer) overnight. Primary antibodies were removed with 3 washes in wash buffer, followed by addition of secondary antibodies (Alexa fluor 488 Goat-anti-mouse; Life Technologies; cat. A11029 or Alexa fluor 594 Goat-anti-rabbit; Life Technologies; cat. A11037) in wash buffer (1:200). Cells were measured by the intensity of fluorescence per cell divided by DAPI-stained nuclei as a percentage.

**Platelet releasate growth factor multiplex immunoassay.** Growth factors contained in the platelet releasate were measured by using high-performance multiplex immunoassays with the Bio-Plex Pro™ Human Cancer Biomarker Panel 1 and 2, (BioRad, UK, Cat. 171AC500M and 171AC600M respectively). This array system includes a blend of magnetic bead-based assays for a number of biomarkers involved in cell division such as Angiopoietin-2, sCD40L, EGF, Endoglin, sFASL, HB-EGF, IGFBP-1, IL-6, IL-8, IL-18, PAI-1, PLGF, TGF- $\alpha$ , TNF- $\alpha$ , uPA, VEGF-A, VEGF-C, VEGF-D, sEGFR, FGF-basic, Follistatin, HGF, sHER-2/neu, sIL-6R $\alpha$ , PECAM-1, PDGF-AB/BB, SCF, sTIE-2, sVEGFR-1, sVEGFR-2 as defined in Supplementary **Table 1**. All the assays were performed using the Bio-Plex 200 system according to the manufacturers' instructions. Heatmap of hierarchical clustering and principal component analysis of Bio-Plex data was performed on the Perseus software (version 1.5.5.3).

**RNA extraction and real-time PCR analysis.** Quantitative PCR was performed as described previously<sup>88</sup>. In brief,  $2.4 \times 10^5$  C2C12 myoblasts were seeded per well of a 6-well plate in SF with or without 10% platelet releasate. After 24 hours cells were harvested in TRIzol (AMRESCO RiboZol™ RNA Extraction Reagent) for RNA isolation and qPCR. Total

RNA was prepared using the EZNA Total RNA Kit I (Omega Biotek, USA). Total RNA (2 µg) was reverse-transcribed to cDNA with RevertAid H MinusFirst Strand cDNA synthesis kit (ThermoFisher Scientific, USA) and analysed by quantitative real-time RT-PCR on a StepOne Plus cycler (Applied Biosystems, UK), using the Applied Biosystems SYBRGreen PCR Master Mix (Thermo Fisher Scientific; cat. 4364344). Primers were designed using the software Primer Express 3.0 (Applied Biosystems, UK). Details of primers are given in **Supplementary Table 2**. Relative expression was calculated using the  $\Delta\Delta C_t$  method with normalisation to the reference genes encoding cyclophilin-B (*Cyp*) and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*)<sup>89</sup>. mRNA levels of *Vegfa165*, *Vegfr1*, *Igf-1*, *Igf-1r*, *Pdgfa*, *Pdgfra*, *Pdgfb*, *Pdgfrb*, *Pax7*, *Myf5*, *Myod*, *Prmt1*, *Ogg1*, *Gadd45g*, *Parp1*, *Ngf*, *Bdnf*, *Ntf3*, *TrkA*, *TrkB* and *TrkC* were measured in cell cultures treated with serum-free, platelet releasate, growth medium or growth medium and releasate for proliferation. Similarly, mRNA levels of *Pax7*, *Myod*, *Pdgfb*, *Vegfa165*, *Myogenin*, *Mhc1*, *Mhc2a*, *Mhc2b*, *Acta1*, *Tmem8c*, *Bex1* and *Igf-1* for growth medium  $\pm$  releasate followed by differentiation medium  $\pm$  releasate.

**Seahorse XFp extracellular flux measurements.** C2C12 myoblasts and isolated satellite cells were seeded at a density of 10,000 cells per well in 8-well XF plates. Cells were pre-incubated under serum-free conditions or treated with 10% releasate, 10% FBS or 10% releasate plus 10% FBS for 24 hours. Prior to the experiment, sensor cartridges were hydrated with XF calibrate solution (pH 7.4), as instructed by the manufacturer's instructions and incubated at 37 °C in a non-CO2 environment for 24 hours. The cell culture medium was replaced with assay medium containing 1mM sodium pyruvate and incubated for one hour in a non-CO2 incubator. Carbonyl cyanide 4- (trifluoromethoxy)phenylhydrazone (FCCP; 5 µM final concentration) and antimycin (2.5 µM final concentration) were diluted in the assay medium. The Seahorse XFp Analyzer (Seahorse Biosciences) was used to measure the oxygen consumption rate (OCR) in real time. Baseline measurements of OCR were taken

before sequential injection of FCCP and antimycin. After three basal assay cycles, FCCP was injected to measure maximal mitochondrial respiration by uncoupling ATP synthesis from electron transport followed by injections of antimycin to measure the non-mitochondrial respiratory rate. Data were normalised to protein levels (BCA; Pierce Biotechnology).

**In Vivo cardiotoxin-induced muscle injury.** On day 1, Crl:CD-1 mice (12 week old) were tail vein injected (IV) either with 100  $\mu$ L platelet releasate or 100  $\mu$ L PBS (n=5 per group). 30 minutes later, mice were injected with a total of 30 $\mu$ L, 50 $\mu$ M *Naja pallida* cardiotoxin (CTX; Latoxan, Valence France) into the *tibialis anterior* (TA) muscle. 24 hours later (day 2), the mice received a second identical intravenous injection of either the platelet releasate or PBS. At 5 days, mice were sacrificed, the TA muscles were collected, immediately frozen and 12 $\mu$ M cryo-sections were processed for immunohistochemistry.

**Statistical analysis.** Data are reported as mean $\pm$ SD. Cell culture experiments were conducted with n=3-9 technical replicates and n=2-3 independent experiments as indicated in figure legends. Statistical differences among experimental groups were determined by one-way ANOVA followed by the Tukey post-hoc test. Differences between two groups were detected by using Student's *t* test. Statistical differences were considered as significant for  $p < 0.05$ . Statistical analysis was performed on the SPSS software (IBM SPSS Statistics version 24).

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## Conflict of interest

The authors declare no conflict of interest.

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## Figure legends

**Figure 1. The effect of platelet agonists and species-of-origin during releasate preparation on C2C12 myoblast proliferation.** (A) A schematic depicting the methods of obtaining platelet releasate or platelet lysate. (B-C) Representative images and quantitative data for C2C12 myoblast proliferation (EdU) and nuclear staining (DAPI) by releasate from platelets activated with collagen, TRAP6 (a PAR1 agonist) or thrombin (x5 magnification, scale bar 200µm). Lysates from mechanically-activated platelets was achieved by sonication (S.L.). Control conditions included serum-free (SF) DMEM ± 10-20% FBS (GM). (D) Total protein concentrations (mg mL<sup>-1</sup>) for the 3 platelet releasates activated with different platelet agonists. (E) Dose-dependent effect of PAR1-activated platelet releasate (i.e. 10, 20, 30% v/v) on C2C12 myoblast proliferation after 24h. Representative images for proliferating C2C12 myoblasts (EdU) and total nuclear staining (DAPI); (x10 magnification, scale bar 200µm). (F) Quantitative data on the effect of human (Human R; stimulated with PAR1) and mouse platelet releasate (Mouse R; stimulated with collagen) on C2C12 myoblast proliferation (EdU). Data are mean±SD (n=3/group, 3 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are \*p<0.05, \*\*\*p<0.001 and #p<0.001 vs. every other group.

**Figure 2. The effect of releasate from different platelet concentrations on C2C12 myoblast proliferation and myoblast gene and protein expression patterns. (A)**

Representative images for C2C12 myoblast proliferation (EdU) and nuclear staining (DAPI) (x5 magnification, scale bar 200µm) in response to physiological (i.e.  $2.5 \times 10^8$ ) and non-physiological platelet concentrations. Quantitative data showed a significant effect of physiological platelet concentrations. Quantitative data showed a significant effect of physiological platelet concentration on C2C12 myoblast proliferation that was comparable to 10% FBS (GM) and supra-physiological platelet concentrations (i.e.  $5-10 \times 10^8$ ). Control conditions included serum-free (SF) DMEM  $\pm$  10% FBS (GM). Data are mean $\pm$ SD (n=3/group, 2 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Statistically significant differences are \*\*\*p<0.001 and # for p<0.001 compared to GM and  $2.5-10 \times 10^8$  respectively. (B) Myoblast gene expression patterns using releasate from physiological platelet concentration (i.e.  $2.5 \times 10^8$ ) by qPCR for *Vegfa165*, *Vegfr1*, *Igf1*, *Igf1r*, *Pdgfa*, *Pdgfra*, *Pdgfb*, *Pdgfrb*, *Pax7*, *Myf5*, *Myod* and *Prmt1* on 10% releasate vs. control (DMEM). Data are mean $\pm$ SD (n=6/group). Statistical analysis was performed by unpaired Student's *t*-tests. Differences are \*\*\*p<0.001, \*\*p<0.01. (C) Immunohistochemical staining of PDGF B, VEGF or IGF-1R $\alpha$  in C2C12 myoblasts cultured with SF, 10% FBS (GM) or 10% R for 24 hours; (x10 magnification, scale bar 200µm, inlay images are x40 magnification with 20µm scale bars). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are \*p<0.05, and \*\*p<0.01 vs. the SF control group.

**Figure 3. Growth factors measured in platelet releasate.** Human platelet suspensions were aggregated using either TRAP6 (a PAR1 agonist), or collagen, or were left unstimulated. Concentrations of specific analytes contained in BIOPLEX ONCO I and II panels were measured in platelet releasate. Platelets from n=6 independent donors were used in this assay. (A) Hierarchical clustering representing the average expression intensity of each analyte per condition (U, unstimulated; C, collagen; T, TRAP6). (B) Principal

Component Analysis (PCA) representing the distribution and grouping of samples considering the analytes measured as a whole.

**Figure 4. VEGFR and PDGFR inhibitors dose-dependently attenuate the effect of releasate on C2C12 myoblast proliferation.** C2C12 cells were treated with either 10% FBS (GM) or 10% Releasate  $\pm$  VEGFR Inhibitor (AAL-993; 23nM, 130nM or 1.30 $\mu$ M) or 10% releasate  $\pm$  PDGFR Inhibitor (Tyrphostin AG 1295; [AG-1295] 250nM, 500nM or 5 $\mu$ M). Control conditions included serum-free (SF) DMEM  $\pm$  10% FBS (GM) or 10% Releasate (R). (A) Representative images for C2C12 myoblast proliferation (EdU) and nuclear staining (x5 magnification, scale bar 200 $\mu$ m) (DAPI). (B) Quantitative data on the effect of VEGFR and PDGFR inhibition show a dose-dependent attenuation of myoblast proliferation with or without releasate treatment. Data are mean $\pm$ SD (n=8/group, 2 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 and #p<0.05 vs. the SF (control) group, ¥p<0.05 vs. the R group.

**Figure 5. Releasate causes C2C12 myoblasts to fail exiting the proliferative phase dependent on the timing of application.** (A) Schematic depicting the experimental setup; C2C12 myoblasts were seeded with either 10% releasate (R) and/or 10% FBS (GM) for 2 days followed by adding differentiation medium (DM; 2% horse serum) every 0.5 days  $\pm$ R. for a total of 9 days culture. Representative images for Myogenin and DAPI (x5 magnification, scale bar 200 $\mu$ m). The myotube fusion index was calculated by Myogenin<sup>+ve</sup> nuclei in myotubes/ DAPI as a percentage. Outcome measures include the nuclei number, number of Myogenin<sup>+ve</sup> nuclei, myotubes (n=3 nuclei/ myotube), myotube thickness and length ( $\mu$ m). Gene expression for *Pax7*, *Myod*, *Pdgfr $\beta$* , *Vegf165 $\alpha$* , *Myogenin*, *Mhc1*, *Mhc2a*, *Mhc2b*, *Acta1*, *Tmem8c*, *Bex1* and *Igf-1* was measured by qPCR for these 3 groups (i.e. GM;DM, GM+R;DM and GM+R;DM+R). (B) Schematic depicting experimental setup, as well as representative images (x5 magnification, scale bar 200 $\mu$ m). C2C12 cells were seeded



with 10% FBS (GM) (GM;DM, GM;DM+R.Day 5-12). For the next 8 days, DM was added daily for every group and releasate added daily with each media change on day 5-12. Outcome measures include the number of myotubes, nuclei, myonuclei, myotube thickness and length ( $\mu\text{m}$ ). Data are mean $\pm$ SD (n=9/group, 3 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, vs. the positive control (GM:DM).

**Figure 6. PDGFR and VEGFR Inhibitors have a detrimental effect on nuclei number, myogenin expression and myotube number during differentiation.** (A) Schematic depicting the experimental setup; C2C12 cells were seeded with either 10% FBS (GM), 10% Releasate (R). After 1 Day of proliferation, media was changed to 2% horse serum (DM) with or without a 5 $\mu\text{M}$  PDGFR Inhibitor (AG-1295) or a 130nM VEGFR Inhibitor (AAL-993). Media was changed daily with inhibitors added on each change. Releasate was added on day 5 only, 1 day before experimental termination (GM;DM+R D.5). (B) PDGFR and VEGFR inhibition in control cultures (GM:DM). (C) PDGFR and VEGFR inhibition in cultures supplemented with releasate on day 5 (GM:DM+R D5 only). (B-C) Representative images for myogenin and DAPI. The myofusion index was calculated by myotube nuclei/ total DAPI as a percentage. Outcome measures include the number of nuclei, myogenin<sup>+</sup> nuclei, myotubes, myotube thickness and length ( $\mu\text{m}$ ). Data are mean $\pm$ SD (n=3/group, 2 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 vs. the positive control (GM;DM).

**Figure 7. Platelet releasate increases myofibre stem cell commitment to differentiation through VEGF and PDGF in a dose-dependent manner.** Murine C57Bl/6J single fibres were isolated from the extensor digitorum longus muscle (EDL) for 48 hours culture in single fibre media (FM), or serum free media (SF) with the addition of releasate (R) either x1 or x2 (every 24 hours) or x3 (every 16 hours) media changes. (A-B) The percentage of muscle

stem cells/ fibre expressing myogenic regulatory factors (MRFs) Pax7<sup>+ve</sup>/MyoD<sup>+ve</sup> (White arrows) for cell activation, Pax7<sup>-ve</sup>/MyoD<sup>+ve</sup> (Red arrows) for commitment to differentiation and Pax7<sup>+ve</sup>/MyoD<sup>-ve</sup> (Green arrows) for quiescence (n=10 mice, 17 EDL muscles; 50-130 fibres quantified per condition (x40 magnification, scale bar 20µm). **(C)** Percent of Pax7<sup>-ve</sup>/MyoD<sup>+ve</sup> cells per total nuclei (DAPI) per fibre. **(D)** Murine C57BL6 single fibres were isolated from the EDL for 48 hours culture in FM or serum free media (SF) with the addition of 10% releasate (R) (every 16 hours) ± PDGFR (AG-1295) or VEGFR (AAL-993) Inhibitors. Quantitative data representing the percentage of muscle stem cell expression per fibre expressing MRFs (upper graph) and total muscle stem cell number (lower graph). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 vs. every other group for Pax7<sup>-ve</sup>/MyoD<sup>+ve</sup> cells, and #p<0.05 for Pax7<sup>+ve</sup>/MyoD<sup>-ve</sup> cells vs. every other group.

**Figure 8. Platelet releasate causes an increase in muscle stem cell proliferation and regulates cell fate through upregulation of Cyclin D1 and Scrib respectively.** **(A)** Representative images from single fibres stained for Cyclin D1, Scrib, MyoD and DAPI (x40 magnification, scale bar 20µm). **(B)** The relative expression (%) of MyoD, Cyclin D1 and Scrib and DAPI for nuclei staining on muscle stem cells. Results are shown for serum-free (SF), Fibre Medium (10% HS; FM), 10% Releasate (R) and FM+R. **(C)** Cyclin D1 and Scrib immunohistochemical expression in C2C12 as a percentage per DAPI for serum-free, 10% FBS growth medium, and 10% releasate conditions. **(D)** Total muscle stem cell number per condition at time-point 48 hours of single fibre isolation *ex vivo*. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 and #p<0.01 vs. every other group. Differences for (B) refer to Cyclin D1/MyoD (double staining) \*\*\*p<0.001.

**Figure 9. Releasate increases muscle stem cell progeny without affecting the relative expression pattern (%) after 72 hours in serum-free conditions.** Male murine C57BL6 single fibres were isolated from the extensor digitorum longus muscle for 72 hours culture in single fibre media (FM), or serum free media (SF) with or without the addition of releasate (R) every 24 hours. **(A)** Representative images for Pax7 and Myogenin staining (x40 magnification, scale bar 20µm). The percentage of muscle stem (satellite) cells per fibre expressing Pax7<sup>+</sup>/Myogenin<sup>+</sup> (white arrows), Pax7<sup>-</sup>/Myogenin<sup>+</sup> (red arrows) for differentiating cells and Pax7<sup>+</sup>/Myogenin<sup>-</sup> (green arrows) for return to quiescence. **(B)** Outcome measures include satellite cell expression pattern (number and percentage per fibre), total number of satellite cells and number of clusters per fibre. **(C)** Standardised rank of the satellite cell progeny for SF, FM and R conditions for T48 and T72 hours. Regression analysis correlating T48 and T72 hour satellite cell progeny numbers revealed different gradient values (i.e. slopes) indicative of the satellite cell proliferation rates. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001 vs. the SF control group.

**Figure 10. Platelet releasate affects myoblast or muscle stem cell metabolism.** **(A)** C2C12 myoblasts were seeded in seahorse plates for 24h in either serum free (SF) ±10% releasate (R), or 10% FBS growth medium (GM) ±10% R. **(B)** Muscle stem cells were isolated from EDL (n=8) single fibres from male C57BL6 mice and cultured for 72 hours in growth media containing 30% FBS and 1.5% chick embryo extract. Cells were then transferred to a seahorse plate in either growth media with GM, R or GM+R. The OCR was measured in response to FCCP and antimycin injections. **(C)** Isolated primary skeletal muscle stem cells from biceps brachii (BB) of C57Bl/6J mice (WT) cultured for 3 days in growth media (GM) or growth media plus releasate (GM+R), before changing to differentiation media for an additional 5 days with or without daily addition of 10% releasate. Representative data outcomes include cell number, myonuclei number, myofusion index and

myotube number. **(D)** Schematic highlighting the beneficial effect of platelet releasate on muscle stem cell proliferation and differentiation. Our findings suggest that platelet releasate increases muscle stem cell commitment to differentiation and promotes skeletal myogenesis through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis. Data are mean $\pm$ SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  for either GM vs. GM+R or SF vs. R and # $p<0.001$  vs. every other group.

**Figure 11. Platelet releasate accelerates skeletal muscle regeneration of tibialis anterior in vivo. (A-B)** Representative images for the identification of regenerating muscle fibres (through the expression of embryonic myosin heavy chain (eMHC), and damaged and dying fibres (identified through the presence of IgG inside muscle fibres on day 5 after cardiotoxin injury and platelet releasate treatment. Scale bar: 100 $\mu$ m. Data are mean  $\pm$ SD. Statistical analysis was performed by Student's t-test. Differences are \*\*\* $p<0.001$  between the cardiotoxin (CTX) and the releasate (CTX + Releasate) group.

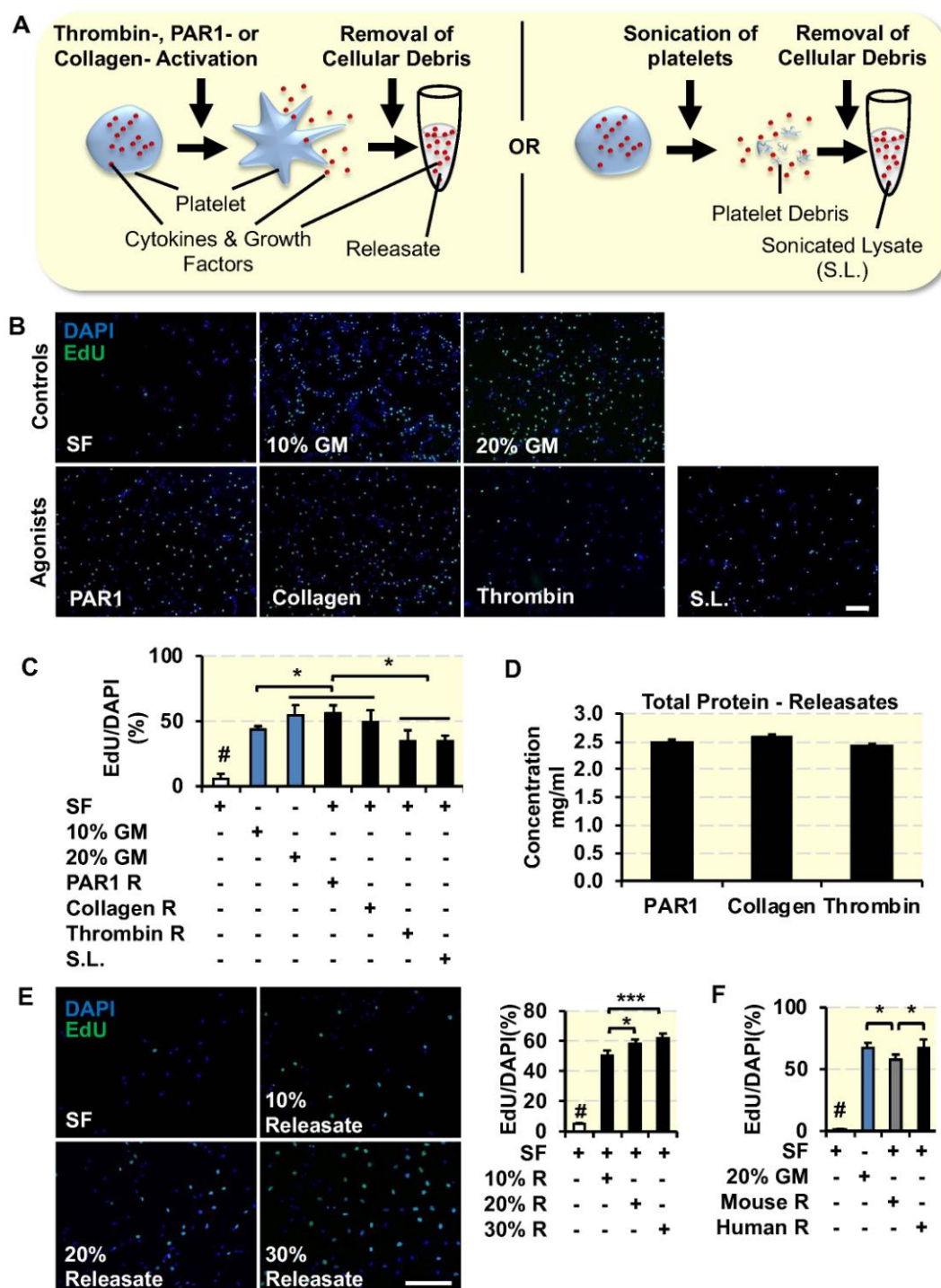


Figure 1



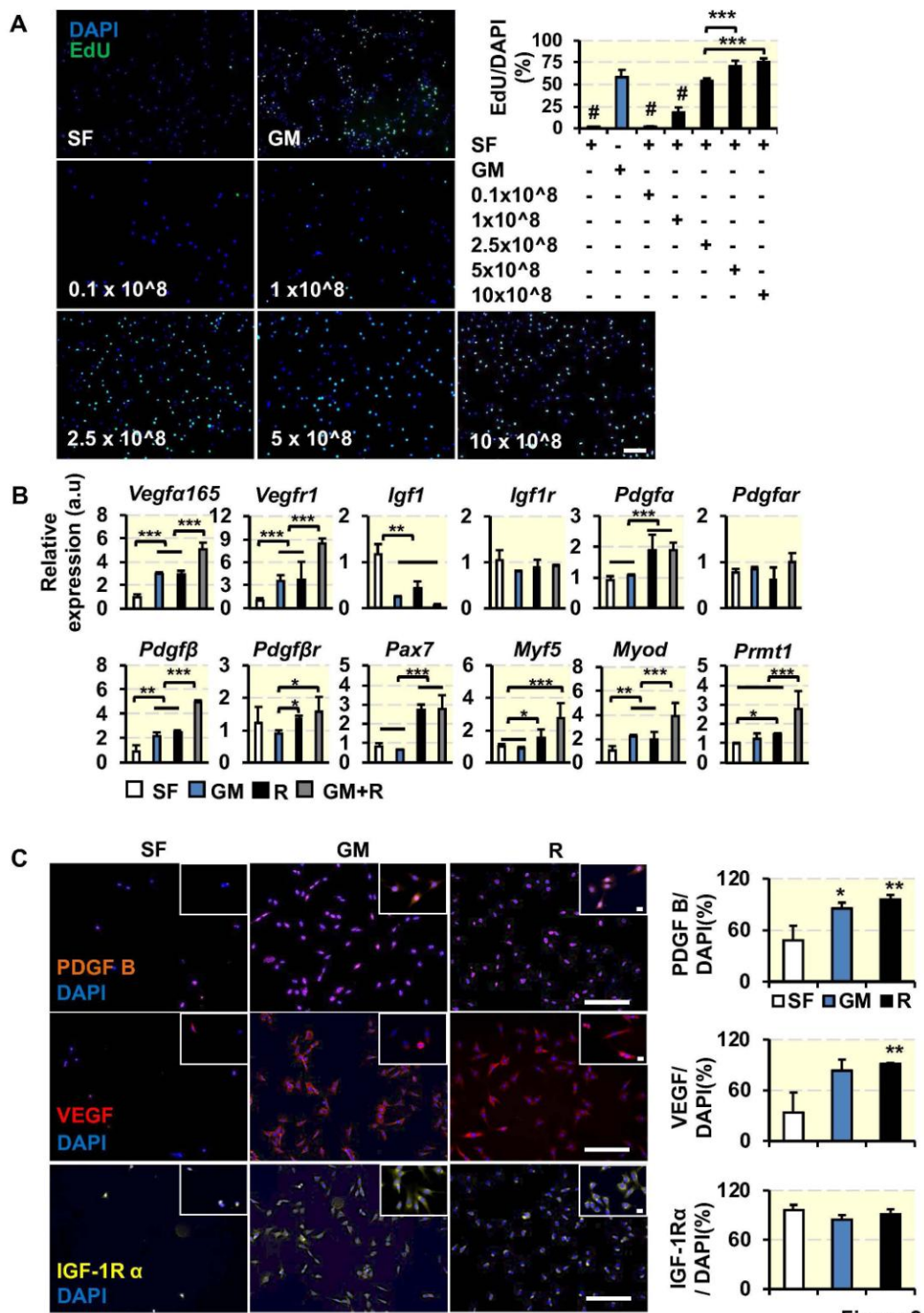


Figure 2

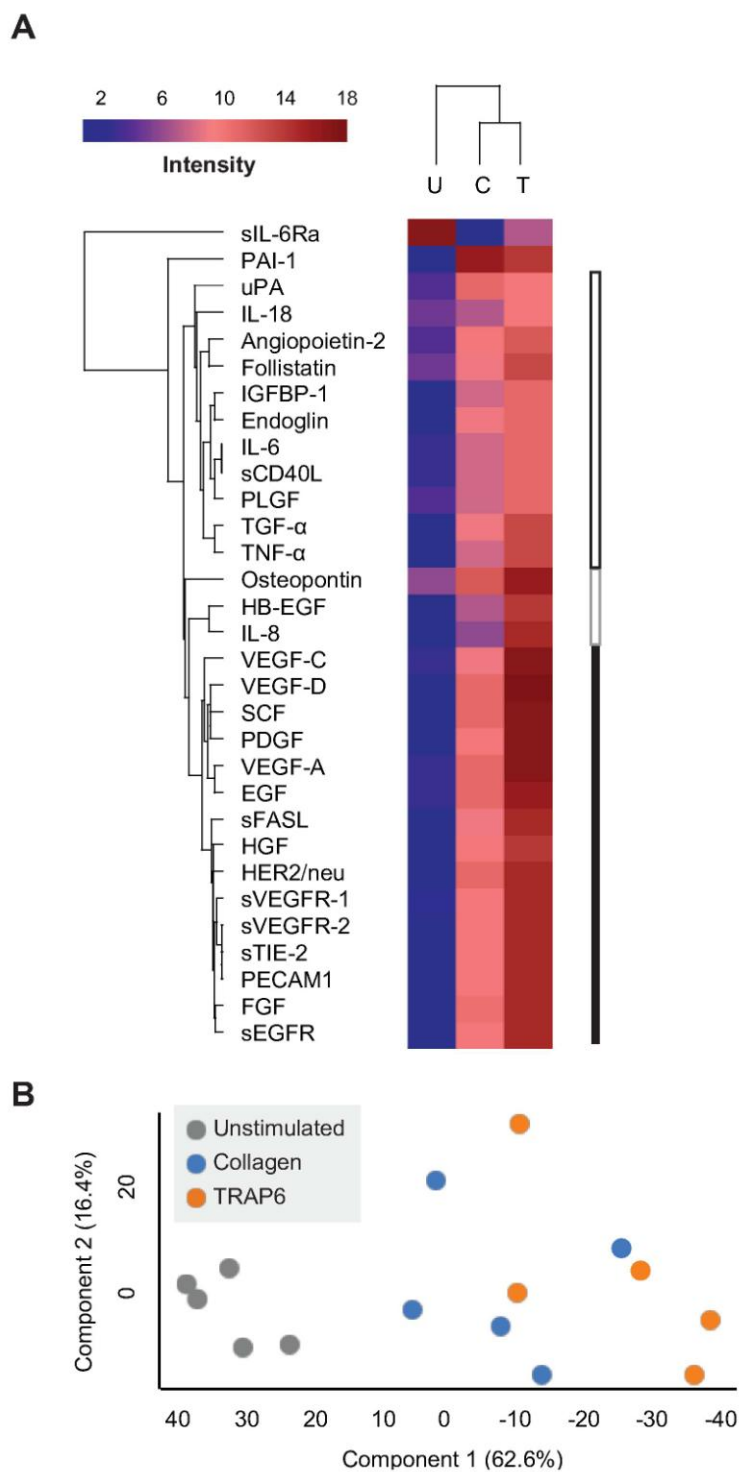


Figure 3

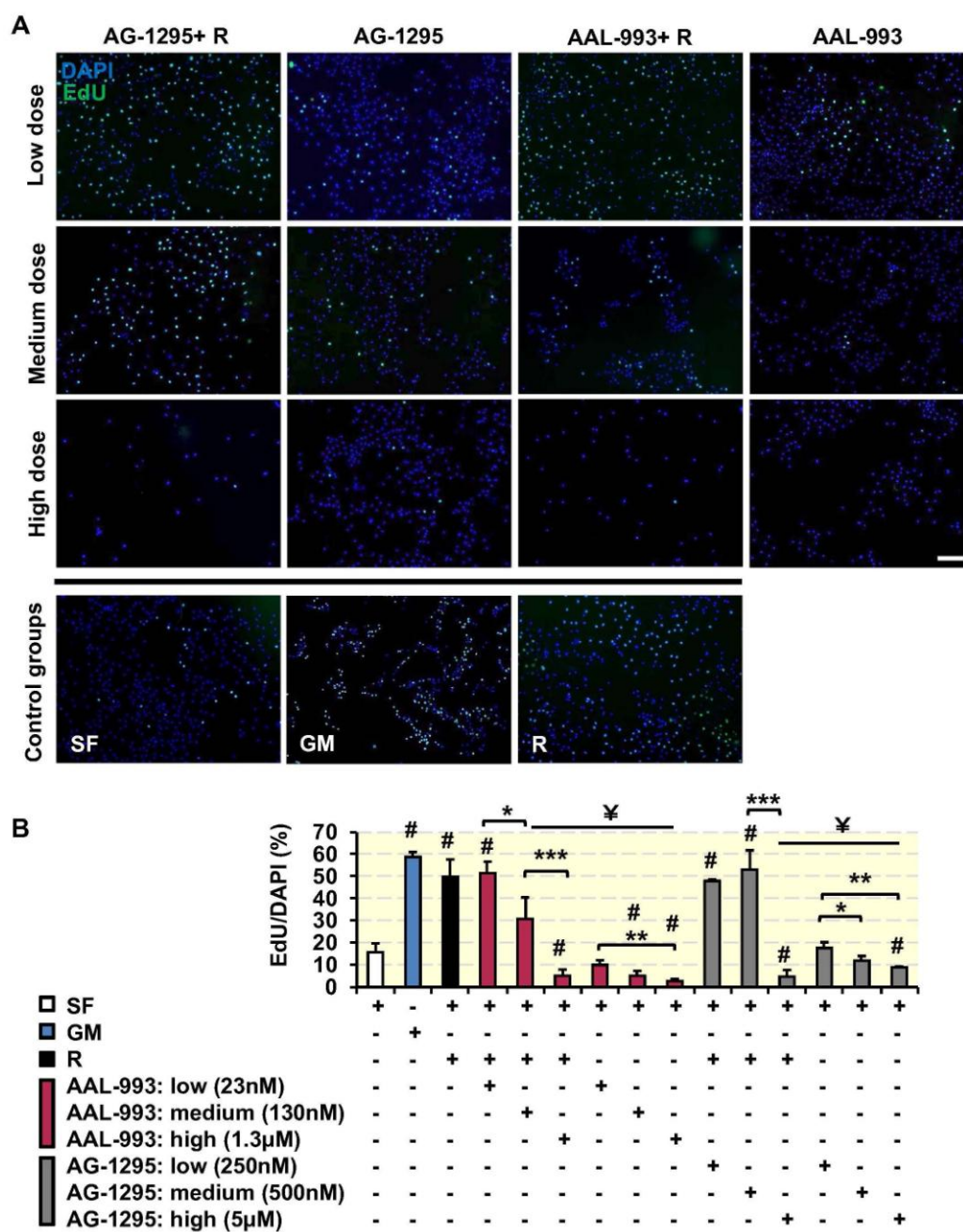


Figure 4



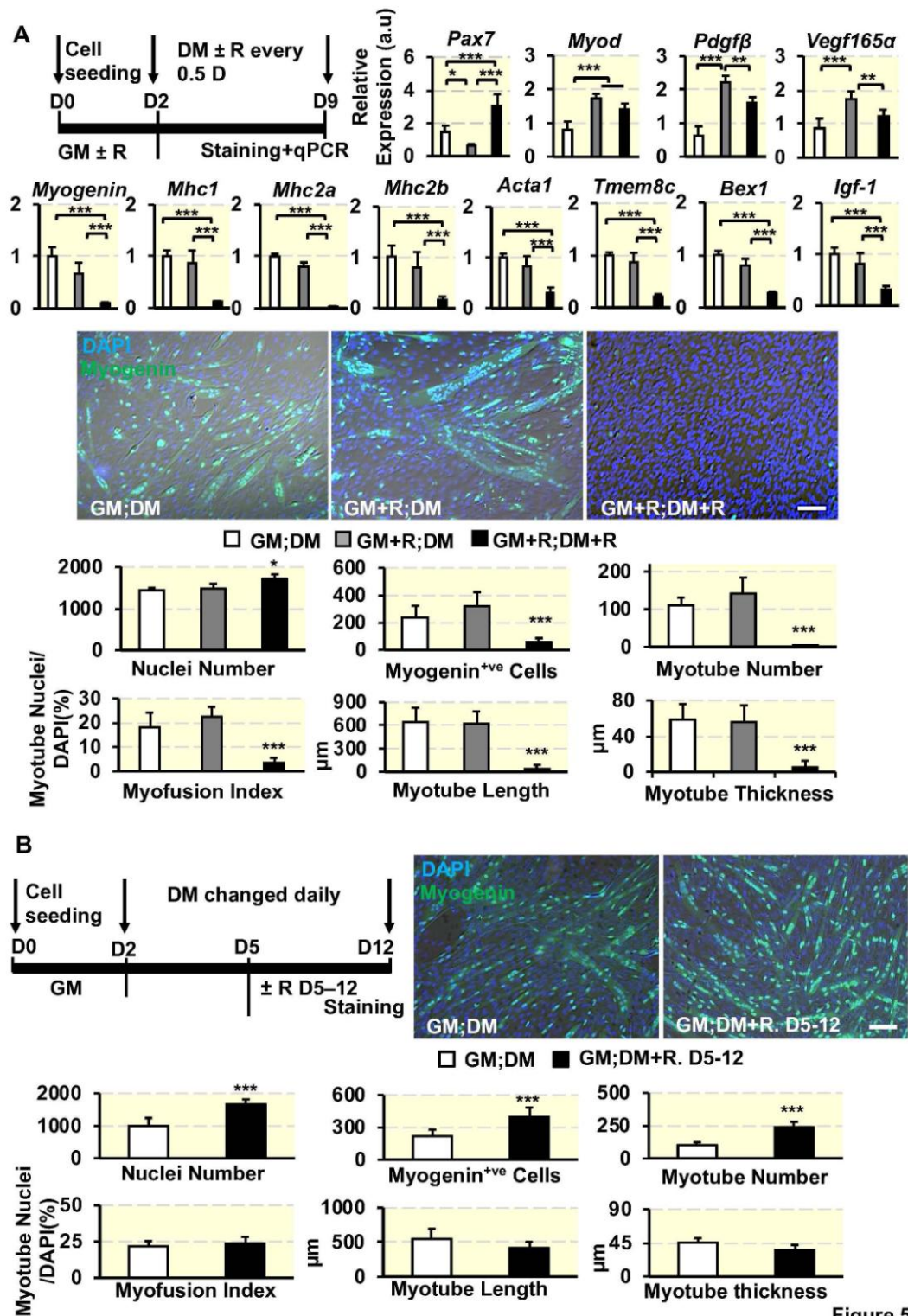
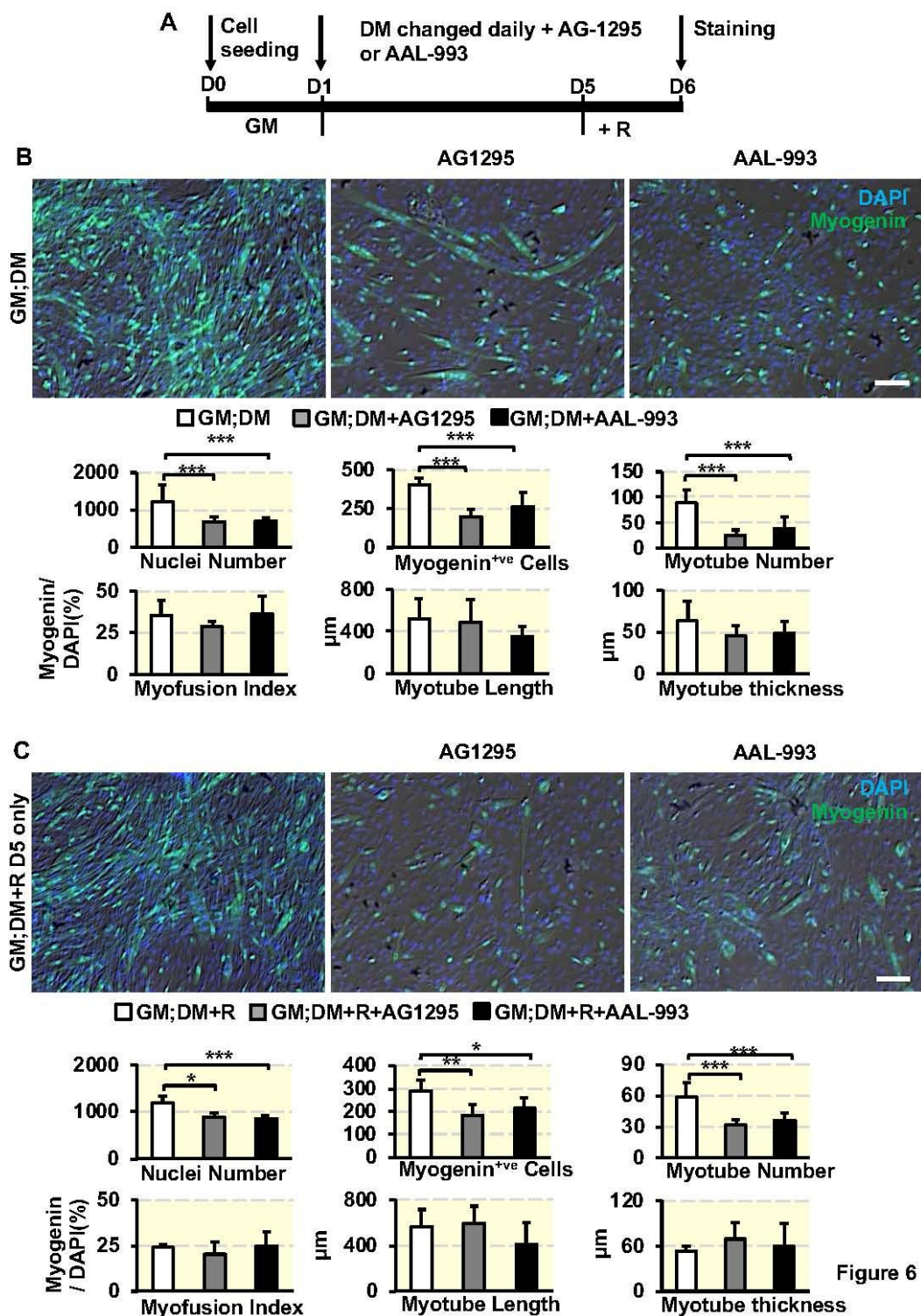
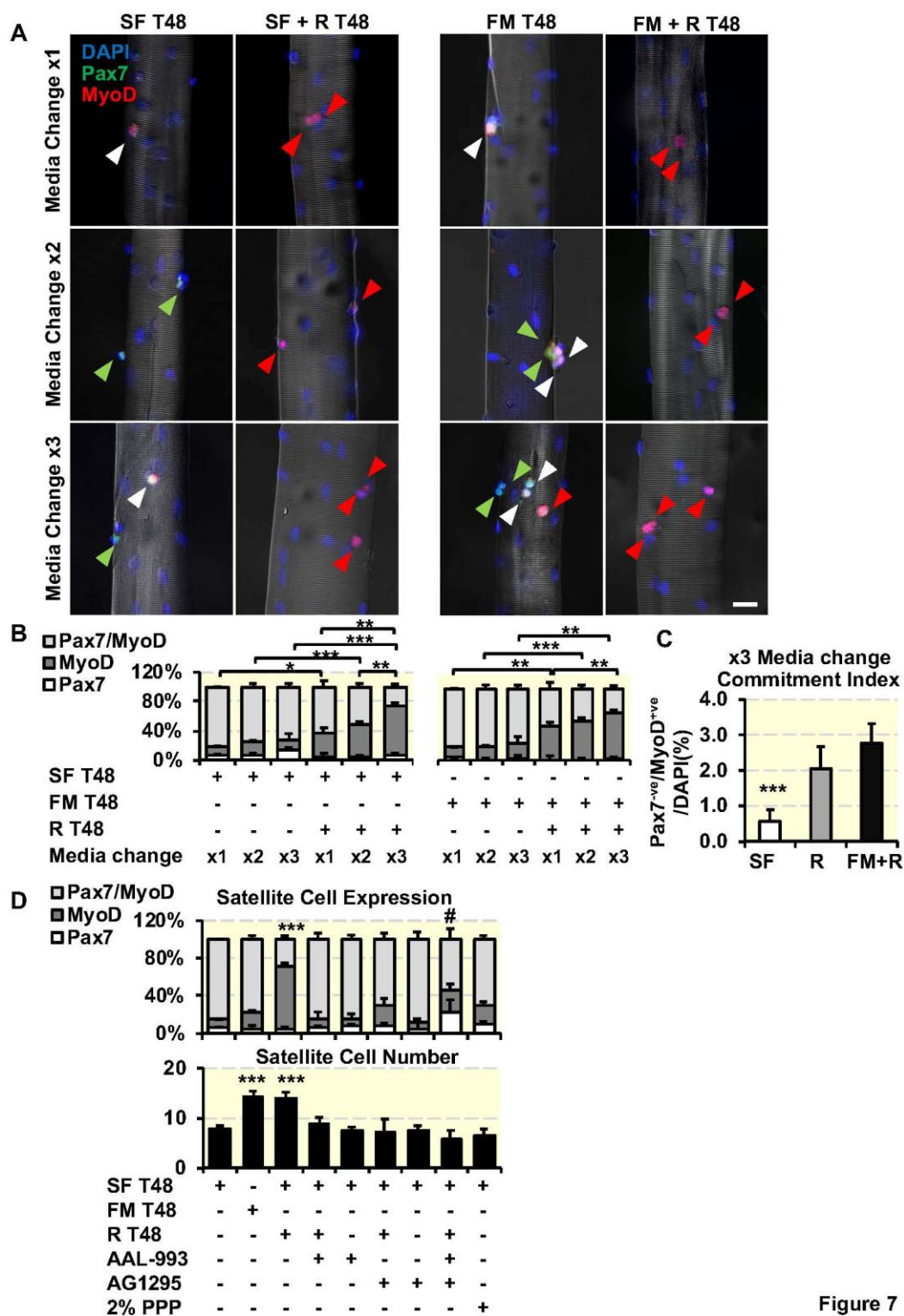


Figure 5







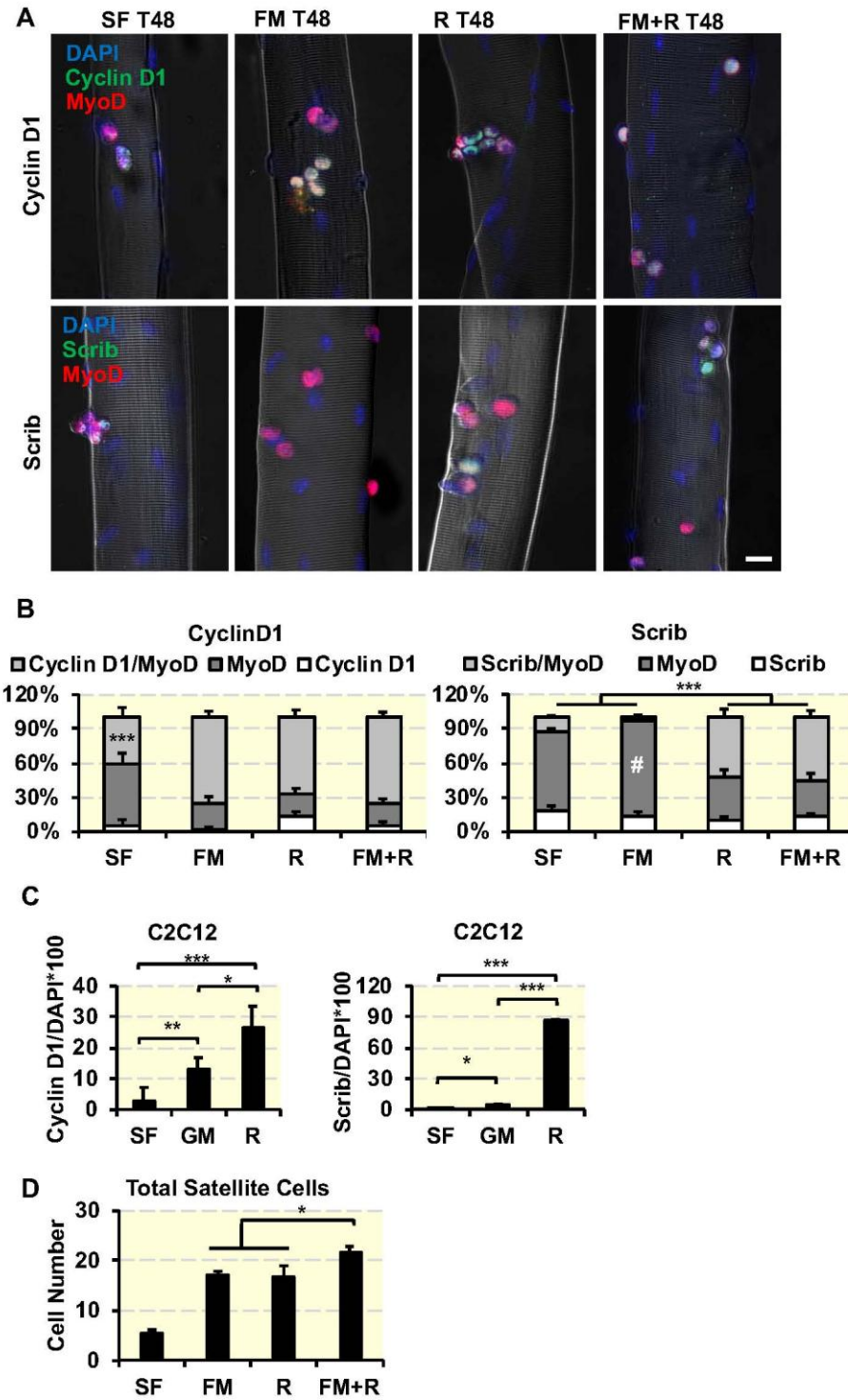


Figure 8

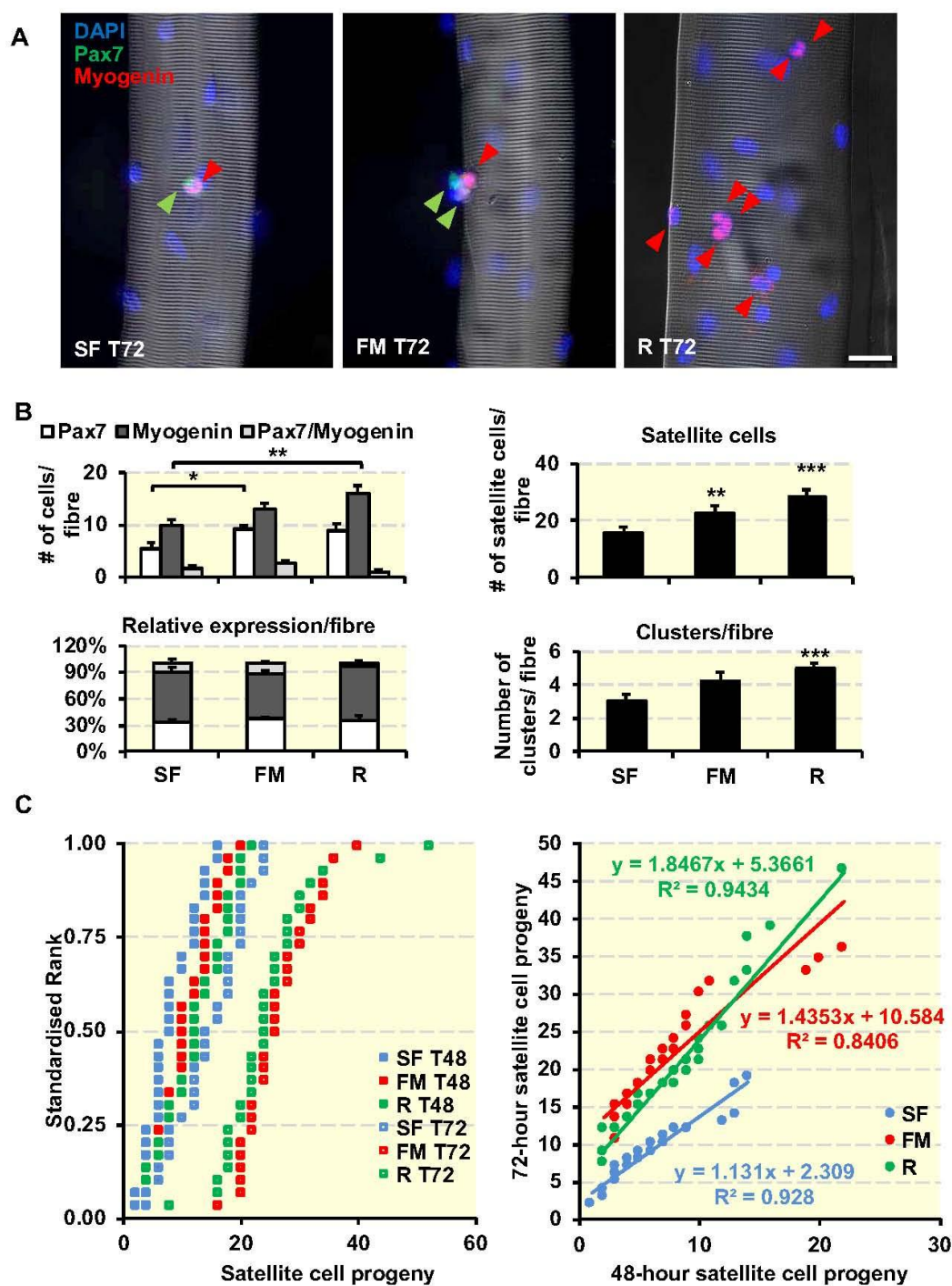


Figure 9

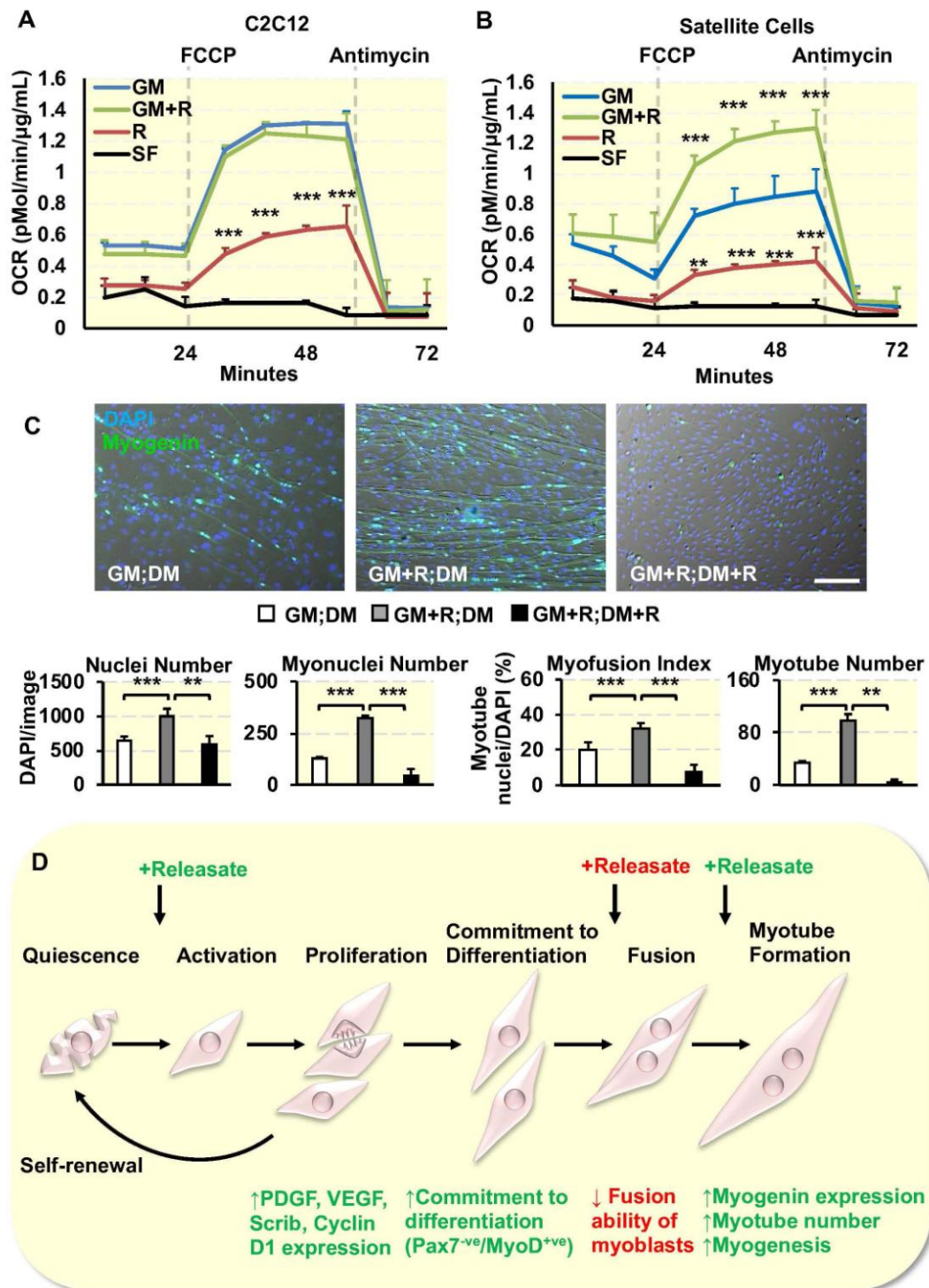


Figure 10

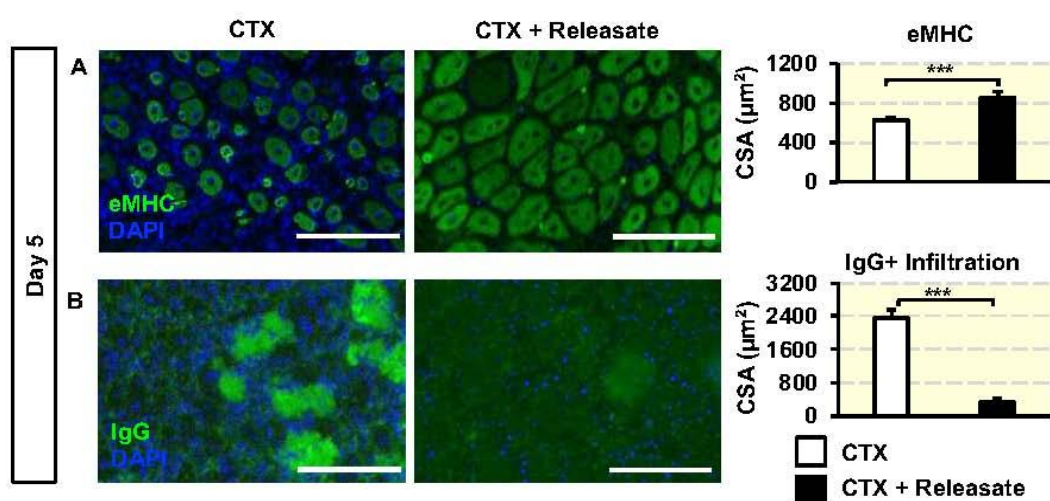


Figure 11